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14498

Effect of Flavor on Nutritive Value of Fats.*

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It has been established that with certain experimental diets important differences in the growth promoting value of different fats can be demonstrated in the rat. Specifically, butter fat has growth promoting properties superior to certain vegetable oils when used in purified diets containing lactose as the sole carbohydrate.^{1,2} An explanation for this fact has been advanced by Deuel and coworkers.³

They contend that the greater growth of weanling rats on a butter diet, where *ad libitum* feeding is practiced, may result from a greater food consumption due to the preference of the rats for butter flavor, namely, diacetyl.

of thiamine, riboflavin, pyridoxine, calcium pantothenate, and choline; and to Winthrop Chemical Company, New York, for crystalline vitamin D₂.

¹ Boutwell, R. K., Geyer, R. P., Elvehjem, C. A., and Hart, E. B., *J. Dairy Sci.*, 1943, **26**, 429.

² Geyer, R. P., Boutwell, R. K., Elvehjem, C. A., and Hart, E. B., *Science*, 1943, **98**, 499.

³ Deuel, H. J., Jr., Movitt, E., and Hallman, L. F., *Science*, 1943, **98**, 139.

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from the Evaporated Milk Association, Chicago; the National Dairy Council, Chicago; and the Wisconsin Alumni Research Foundation. We are indebted to Merck and Company, Rahway, N.J., for supplies

van Niel *et al.*⁴ isolated diacetyl and showed it to be the characteristic flavoring substance in butter; present evidence indicates that the flavor of superior sweet-cream butter is mainly due to diacetyl.⁵ Although work done two years ago at this station had indicated to us that diacetyl was not a factor influencing the relative nutritive value of fats, the experiment was repeated twice, and the data are presented here.

The butter fat was prepared by decantation and filtration of melted (60°C) fresh unsalted sweet-cream butter obtained from the University creamery. The chromatographed butter fat was prepared from this product as follows: 100 g of the fat were dissolved in 400 ml of redistilled petroleum ether (b.p. 45-60°C) and passed through 200 g of a mixture of equal parts of Superfiltrol[†] and Super-Cel contained in a Pyrex adsorption column 35 cm in length and 3 cm in diameter with a 500 ml reservoir at the top end. Construction was such that compressed air of moderate pressure could be used to facilitate percolation. Following the Superfiltrol treatment, the solution was passed through the column packed with 200 g of freshly ignited aluminum oxide (minus 80 mesh). The petroleum ether was completely removed under vacuum, a stream of nitrogen being used to sweep out the last traces of solvent. By this procedure most of the non-saponifiable matter including the diacetyl was removed, leaving practically pure triglycerides. Thus a colorless product free from all traces of butter flavor was obtained.

The colorimetric method of Prill and Hammond,⁶ with measurements in the Evelyn colorimeter, was used to determine the diacetyl content of the original butter fat. The value found, 0.00006%, is in agreement with those reported by Schmalfuss and Barthmeyer,⁷ and

Davies,⁸ but lower than the 0.0002 to 0.0004% reported by van Niel *et al.*⁴ Little difference in diacetyl content was found between natural whole butter and the butter fat itself. The chromatographed butter fat gave no detectable color for diacetyl.

Weanling albino male rats of the Sprague-Dawley strain were fed *ad libitum* a diet of lactose 48, casein (fat-free) 20, salts IV⁹ 4, and fat 28%. The following amounts of vitamins in milligrams were added per 100 g of ration: thiamine 0.5, riboflavin 0.5, niacin 0.625, pyridoxine 0.625, calcium pantothenate 5.0, *p*-aminobenzoic acid 30.0, inositol 100, choline 250, β -carotene 0.56, α -tocopherol 2.25, calciferol 0.014, and 2-methyl-1,4-naphthoquinone 0.21. Four diets were made up, each containing one of the following as the fat portion: (1) butter fat, (2) corn oil, (3) chromatographed butter fat, and (4) corn oil containing 0.0005% diacetyl.

The growth rate and food consumption of 12 rats were followed on each of the 4 diets over a 6-week period. These data are given in Table I. The rats on the butter fat and the chromatographed butter fat regimes grew at a normal rate and were of good outward appearance. The rats on the diets containing either corn oil or corn oil plus diacetyl were inferior animals, exhibiting diarrhea during

TABLE I.

Average Gain and Food Intake During the 6-week Period. Each figure for the grams gained represents the average of 12 male albino rats. Each figure for the food intake represents the average based on the average of 2 groups of 6 rats each.

Group	Avg gain, g	Avg daily food intake, g
1. Butter fat-lactose	145	9.9 ($\pm$ 0.2)
2. Corn oil-lactose	110	7.7 ($\pm$ 0.3)
3. Chromatographed butter fat-lactose	152	9.7 ($\pm$ 0.3)
4. Corn oil-lactose plus 0.0005% diacetyl	124	8.1 ($\pm$ 0.3)
5. Butter fat-dextrose	186*	11.2 ($\pm$ 0.2)*
6. Corn oil-dextrose	183*	11.2 ($\pm$ 0.1)*

* Figure represents the average of 2 groups of 6 male rats each run simultaneously with the lactose groups, and 1 group of 6 male rats run 8 weeks previously, making a total of 18 animals.

⁸ Davies, W. L., *Food Manufacture*, 1933, **8**, 346.

⁹ Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 657.

⁴ van Niel, C. B., Kluyver, A. J., and Dery, H. G., *Biochem. J.*, 1929, **210**, 234.

⁵ Associates of L. A. Rogers, *Fundamentals of Dairy Science*, Reinhold Publ. Corp., New York, 1935, 366.

[†] The Superfiltrol was obtained from the Filtrol Corporation, 315 West Fifth Street, Los Angeles.

⁶ Prill, E. A., and Hammer, B. W., *Iowa State College J. Sci.*, 1938, **12**, 385.

⁷ Schmalfuss, H., and Barthmeyer, H., *Z. physiol. Chem.*, 1928, **176**, 282; *Biochem. Z.*, 1929, **216**, 330.

the first 6 to 12 days of the experiment, followed by mildly scaly paws and blood-stained noses and fur at 3 weeks. The fur coat of the animals in these 2 groups was generally poor from the first week and one case of extreme abdominal alopecia was noted at 5 weeks. The superiority of butter fat over corn oil (Table I, Groups 1 and 2) with respect to weight gain and increased food consumption was again confirmed. It is obvious that these relationships and values were practically unchanged by the removal of diacetyl from butter or by the addition of that compound to corn oil (Groups 3 and 4).

A ration identical to the one outlined above but containing dextrose as the sole carbohydrate in place of the lactose was fed with butter fat and corn oil. The results are included in Table I. With dextrose as the sole source of carbohydrate in the ration, no difference was found between the growth rates or food intake of rats fed butter fat compared with those fed corn oil. Comparison of the results of the dextrose experiment with those of the lactose experiment show that growth of the animals on the former regime was greater than that on the latter, the difference being very marked in the case of the corn oil groups.

Discussion. From these data we conclude that on the rations investigated a flavor factor such as diacetyl or any substance removed from butter fat by chromatographing as

described has little effect on the food consumption and growth of the male albino rat. There are no data in the literature which indicate that by improving the taste or flavor of the diet the growth and food consumption of the rat have been increased. Off flavor and rancidity are known to have an adverse effect. On purified diets the true explanation for the superior growth-promoting value of one substance over another must lie in the existence of specific compounds in that superior nutrient which results in a more favorable physiological response such that the animal grows at a faster rate and hence consumes more ration. Paired feeding penalizes the superior food and ultimately obliterates the difference as measured by the growth rate and well-being of the experimental animal.

Summary. With *ad libitum* feeding and with lactose as the sole carbohydrate, the growth and appearance of albino male rats fed butter fat was superior to that of animals fed corn oil. Removal of the flavoring agents from butter fat by chromatographing or the addition of one such agent, diacetyl, to corn oil had little effect on the comparative nutritive value of the two fats. Rats fed butter fat or corn oil on rations containing dextrose as the sole carbohydrate grew at equal rates and were of normal appearance. Flavor played no part in these dextrose experiments.

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Therapeutic Effects of Forbisen and of Toluidine Blue on Experimental Typhus.

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The therapy of rickettsial infections is almost entirely symptomatic. Various sulfonamides¹ have been used to treat patients, but their usefulness appears to be limited to the treatment of pneumonias or other secondary

bacterial infections in the late stages of the disease. Neoarsphenamine in metaphen,² atebtrin-plasmochin,³ and atebtrin-calcium⁴

² Baker, G. E., *Ann. Int. Med.*, 1942, **17**, 247.

³ van Meerendonk, Piet, *Deut. Militärärzt.*, 1942, **7**, 283. Abstracted by Megaw, J. W. D. M., *Trop. Dis. Bull.*, 1942, **39**, 679.

¹ Wohlrab, V. W., *Klin. Wchnschr.*, 1942, **31**, 445.

mixtures have been employed in therapy, and favorable results have been reported. Further work will be required to assess their value accurately.

In the present study a large number of compounds were investigated for therapeutic activity in mice infected with murine typhus. Murine typhus infections in mice have been described recently by Gildermeister and Haagen⁵ and Pang and Zia.⁶ Yolk sacs of developing eggs infected by the Cox⁷ technic contain sufficiently large numbers of rickettsiae to kill mice by a toxic action within 2 to 8 hours after intraperitoneal injection.⁵ The dose, however, can be adjusted so that the mice are not affected by the toxin but die 4 to 5 days later of rickettsial infection. Mice are as susceptible to the toxin of epidemic strains as that of the murine typhus, but they are completely resistant to infection by epidemic strains.

Materials and Methods. Pools of typhus-infected yolk sac material diluted to a final concentration of 20% in broth were stored in flame-sealed ampoules in the CO₂ icebox. Single ampoules containing 5 cc of material were removed for each experiment. The murine-infected yolk sac suspension diluted to a final concentration of 0.33 to 1.2% usually killed 80 to 100% of the control mice when injected intraperitoneally in 0.5 cc quantity. Compounds being tested were fed in a 1% concentration in finely ground fox chow. No other food was given. Water was supplied by water bottles. The mice were kept in glass jars in groups of 5. Twenty mice were usually used in testing each compound, and a similar group of controls was infected. The feeding of each test drug was started 4 to 24 hours before the infection was given. It was hoped that slight degrees of activity would be detected by this procedure. Several ani-

mals dying in the treated and control groups were autopsied, and smears of peritoneal fluid made and examined microscopically to verify the presence of rickettsia. The Wilmington strain of murine typhus was used throughout except for a few experiments with the Breinl epidemic strain. The results are given below.

Forbisen. Forbisen* was found to possess moderate activity. It was necessary to feed it in a concentration of 2.5% in the diet to produce a significant recovery rate among the treated mice. In a typical experiment 14 of 24 infected mice survived when fed 2.5% forbisen. All of 20 control mice died of the infection.

Toluidine blue. Toluidine blue was very efficacious in preventing deaths from typhus. In an early experiment 40 mice were fed fox chow containing 1.5% toluidine blue. These, along with 20 control mice, were infected 24 hours after feeding was begun. Two treated mice were killed on the 4th day. Of the remaining 38 mice, 27 recovered. There were 4 survivors in the group of 20 control animals. The animals treated with toluidine blue usually showed few or no signs of illness. Deaths usually occurred later than in the untreated group. In the above-described experiment half of the control mice died on or before the 4th day of infection. Three treated mice died in the same interval. The optimum dosage of toluidine blue appeared to be supplied when concentrations of 0.25 to 0.75% were fed in the diet. This represented an ingestion of about 5 to 20 mg a day per mouse. Larger or smaller doses in the diet were often accompanied by an increased mortality. Therapeutic activity could be demonstrated when toluidine blue was administered as late as 24 hours after the infection.

Toluidine blue has a marked action against the Wilmington murine strain *in vitro*. It was added to 5 different concentrations of infected yolk sac suspension to make a final concentration of 1:10,000 toluidine blue. The same quantity of distilled water was added to the infected yolk sac suspensions used as controls. Both sets of tubes were placed in ice water

⁴ van Meerendonk, Piet, *Deut. Militärärz.*, 1942, **7**, 541. Abstracted by Megaw, J. W. D. M., *Trop. Dis. Bull.*, 1943, **40**, 237.

⁵ Gildermeister, E., and Haagen, E., *Deutsche Med. Wchnschr.*, 1940, **66**, 878.

⁶ Pang, K. H., and Zia, S. H., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 76.

⁷ Cox, H. R., *Pub. Health Reports, U.S.P.H.S.*, 1938, **53**, 2241; 1940, **55**, 110.

* Supplied by Mr. W. W. Allen of the Dow Chemical Company.

TABLE I.
Effect of Administering Toluidine Blue at Various Times before and after Infecting Mice with Typhus.

	Feeding of 0.5% toluidine blue in diet started					Control
	24 hr prior to infection	At time of infection	24 hr after infection	48 hr after infection*	72 hr after infection	
No. mice infected	19	20	20	20	20	20
Survivors	14	15	10	1	4	2
% surviving	74	75	50	5	20	10

* Some mice in this group had died and the rest were sick by the time toluidine blue administration was started. Feeding started at 48 hours or later resulted in ingestion of insignificant quantities of toluidine blue.

TABLE II.
The *in vitro* Effect of Toluidine Blue on Rickettsial Toxicity and Infectiousness for Mice.*

% conc. yolk sac, 0.2 cc injected i.v.	Mice surviving injection			
	Murine strain in ice water 1 hr		Epidemic strain incubated 37°C 1 hr	
	Control	Toluidine blue 1:10,000	Control	Toluidine blue 1:10,000
10	0/6*	0/6		
5	0/6	1 (1)/6	0/6	0/6
2.5	0/6	6 (3)/6	0/6	6/6
1.25	1 (1)/6	6 (1)/6	5/6	6/6
0.62	6 (6)/6	6/6	6/6	6/6
Totals:				
No. injected	30	30	24	24
Survived toxin	7	19	11	18
Survived toxin and infection	0	14		

* Open numbers in numerator represent mice surviving toxin. Numbers in numerator in parentheses represent mice surviving toxin but dying of infection. Denominator equals number injected.

for 1 hour before their contents were injected intravenously into mice (Table II). There was a reduction in both the toxin and the infectious titer of the rickettsial suspensions with added toluidine blue. Similar treatment of suspensions of yolk sac infected with the Breinl epidemic strain resulted in no decrease in toxicity. If the contact between the rickettsia of this strain and the toluidine blue in ice water was prolonged to 3 hours, or if the mixture was incubated at 37°C for 1 hour, some reduction in the amount of toxin resulted.

Cotton rats⁸ infected with either the murine or the epidemic strain of typhus by intracardial inoculation have been treated with toluidine blue as well as with forbisen. No

evidence of significant therapeutic activity was obtained.

Discussion. The therapeutic efficiency of toluidine blue in treating typhus-infected mice was in marked contrast with the poor results obtained when cotton rats were treated. The latter animals ate irregularly and mostly at night. This may have made the dosage of any drug fed in the diet quite erratic and thus have contributed to the poor results. No attempt to determine the level of toluidine blue in blood was made. It is possible that the rapid dissemination of the organisms following intracardial inoculation of rickettsia may have overwhelmed the therapeutic activity of toluidine blue.

Summary. Two compounds, toluidine blue and forbisen, have been found to possess therapeutic action against murine typhus infections in mice.

⁸ Snyder, J. C., and Anderson, C. R., *Science*, 1942, 95, 23.

Action of Penicillin and Other Antibiotics on *Treponema Pallidum*.

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The demonstration that penicillin is effective in the treatment of syphilis¹ emphasizes the need for an *in vitro* test for the action of penicillin* and other antibiotic agents on the *Treponema pallidum*. Preliminary tests employing penicillin *in vitro*, with 240 Oxford units per 0.8 ml, failed to reveal any action on the spirochetes. This was in spite of the fact that the concentration employed, which was equivalent to 300,000 O.U. per kg, was considerably greater than appears to be required to protect rabbits from infection. This may be due to the fact that the test period is necessarily brief, as the spirochetes become immotile within a few hours outside the body under optimal conditions, and to the fact that the only criterion for the action of a drug in such a test is the rendering of the spirochetes immotile. Tests were, therefore, performed in which the drug was used in still higher concentrations, up to 3200 O.U. per 0.8 ml. These gave satisfactory results.

Employing the technic of Eagle,² the testes of rabbits in the stage of acute orchitis following inoculation with the Nichols strain were ground and suspended in a mixture of equal parts of normal rabbit serum and physiological saline solution. This was centrifuged at 1500 r.p.m. for 10 minutes and the supernate withdrawn for use in the tests. Serial dilutions of the antibiotic agent in the solvent required for the preparation of the original solution were mixed with equal volumes of the suspension and placed in an atmosphere of hydrogen at approximately 22°C for 2 hours. Dark field examinations were then made and the

percentage of immotile spirochetes determined. Control tubes containing only suspension and the solvent employed for the antibiotic agent were also included in the tests. The percentage of spirochetes immobilized by the antibiotic agent was calculated by means of the following formula:

$$\frac{\text{Drug \% Immotile} - \text{Control \% Immotile}}{100 - \text{Control \% Immotile}} \times 100$$

Besides the method of computing the results, the only other modification of Eagle's technic consisted of putting mineral oil around the edges of the coverslip, which prevented the formation of currents and so aided in determining true motility of the organisms when this was slight.

The mean of the results of the tests with penicillin dissolved in physiological saline solution is shown by the solid line in Fig. 1. This indicates that under the conditions of the test there is a relatively sharp increase in the action on the spirochetes between a concentration of 800 and 1600 O.U. per 0.8 ml.

The results obtained with certain other antibiotic agents are given in Table I. Gliotoxin[†] had a marked spirocheticidal action, while aspergillilic acid³ and bromo-aspergillilic acid⁴ were less effective. Fumigacin⁵ showed the least activity.

During certain studies with penicillin on the prophylaxis of syphilitic skin infections in rabbits, it was noted that some chancres

¹ Mahoney, J. F., Arnold, R. C., and Harris, A., *Am. J. Pub. Health*, 1943, **33**, 1387.

* Part of the penicillin used in this study was allocated by the War Production Board for company research.

² Eagle, Harry, *J. Pharm. and Exp. Therap.*, 1940, **69**, 342.

[†] Obtained through the courtesy of Dr. J. D. Dutcher, Division of Organic Chemistry, The Squibb Institute for Medical Research.

³ White, E. C., *Science*, 1940, **92**, 127.

⁴ Dutcher, J. D., Rake, G., Wintersteiner, O. P., and Jones, H. P., to be published.

⁵ Menzel, A. E. O., Wintersteiner, O. P., and Hoogerheide, J. C., *J. Biol. Chem.*, 1944, **152**, 419.

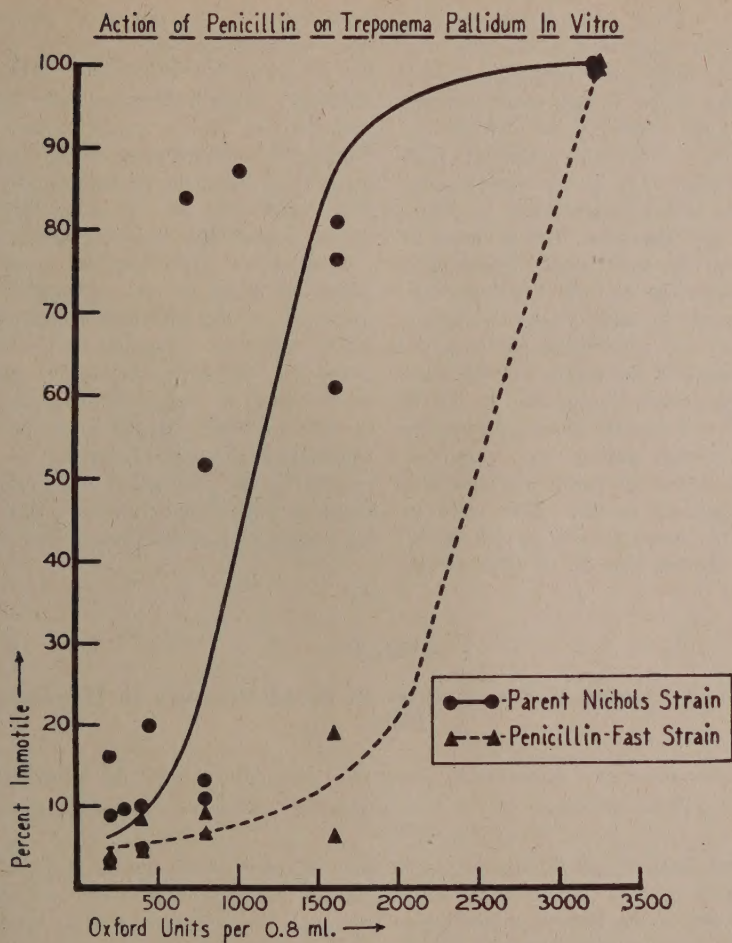


FIG. 1.

TABLE I.

Action of Aspergillie Acid, Bromo-Aspergillie Acid, Fumigacin, and Gliotoxin on *T. pallidum* in vitro.

Agent	mg/0.8 ml	Solvent	% immobilized by agent
Aspergillie Acid	.5	1% NaHCO ₃	100
	.1		10
	.05		13
	.01		6
Bromo-Aspergillie Acid	.5	1% NaHCO ₃	26
	.1		21
	.05		15
	.01		2
Fumigacin	2.0	0.3% NaHCO ₃ and 0.15% Na ₂ CO ₃	34
	1.0		6
	0.4		0
Gliotoxin	.04	2% Alcohol	93

developed late. These lesions were smaller than is usual. A suspension of the popliteal lymph nodes of one of these rabbits was used to inoculate the testes of two other rabbits, one of which later developed an orchitis less severe than that ordinarily observed with the Nichols strain. The testes were ground and an *in vitro* test was performed to determine whether any alteration had occurred in the resistance of the spirochetes to penicillin. Using this suspension another intratesticular passage was made in rabbits and the action of penicillin on the spirochetes again determined. The mean of the results of both tests is shown by the broken line in Fig. 1. These clearly show that the strain obtained from the insufficiently treated rabbit was more resistant to the action of penicillin than was the original Nichols strain. The tests in which 1600 O.U. were present in 0.8 ml of final mixture showed that 62 to 81% of the

spirochetes of the unaltered Nichols strain were immobilized, while only 6 to 18% of the penicillin-fast spirochetes were rendered immotile. This finding indicates that in the treatment of human syphilis, care must be exercised to administer a sufficient quantity of penicillin to cure the patient rapidly. Otherwise, there will be the possibility of producing a penicillin resistant strain.

Summary.—The test for spirocheticidal action of drugs *in vitro* (Eagle²) has been used with certain antibiotic substances. Penicillin, originally presumed to be inactive *in vitro*, has proved to be active under these conditions in the relatively high concentration of 800 to 1600 Oxford units per 0.8 ml. Inadequate therapy of syphilis in a rabbit resulted in the production of a more resistant strain of *Treponema pallidum* and emphasizes the importance of adequate initial therapy.

14501 P

Influence of Vitamin K Preparations on Blood Pressure in Hypertensive Rats.*

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While we have been occupied with the preparation of kidney extracts capable of lowering the blood pressure, it has become increasingly clear that the hypertension resulting from inadequate blood supply to the kidney might be due to faulty deamination of certain amino acids. Holtz *et al.*¹ found that in anaerobic conditions, such as may exist in renal ischemia, decarboxylation without subsequent deamination can occur, thus leading to the accumulation of pressure amines, and Bing and co-workers^{2,3} demonstrated that experi-

mental hypertension can be produced by injection of amino acids into the ischemic kidney of cats. Under normal aerobic conditions, on the other hand, such amines do not accumulate since they are readily destroyed by the catalytic action of amino oxidase. Friedman, Soloway, Marrus and Oppenheimer⁴ demonstrated that certain quinones, which may inactivate pressure amines, may have well-defined blood pressure reducing qualities in hypertensive rats. Grollman and Harrison,⁵ found some blood pressure lowering substance in body and liver oils of fish and assumed that this substance might also be a quinone. It was, therefore, suggestive to investigate the

* The authors wish to express their gratitude to the American Philosophical Society for financial support from the Daland Fund.

¹ Holtz, P., Heise, R., and Luedtke, K., *Arch. Exp. Path. u. Phar.*, 1938, **191**, 87.

² Bing, R. J., *Am. J. Phys.*, 1941, **132**, 497.

³ Bing, R. J., and Zucker, M. B., *J. Exp. Med.*, 1941, **74**, 235.

⁴ Friedman, B., Soloway, S., Marrus, J., and Oppenheimer, B. S., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 195.

⁵ Grollman, A., and Harrison, T. R., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 162.

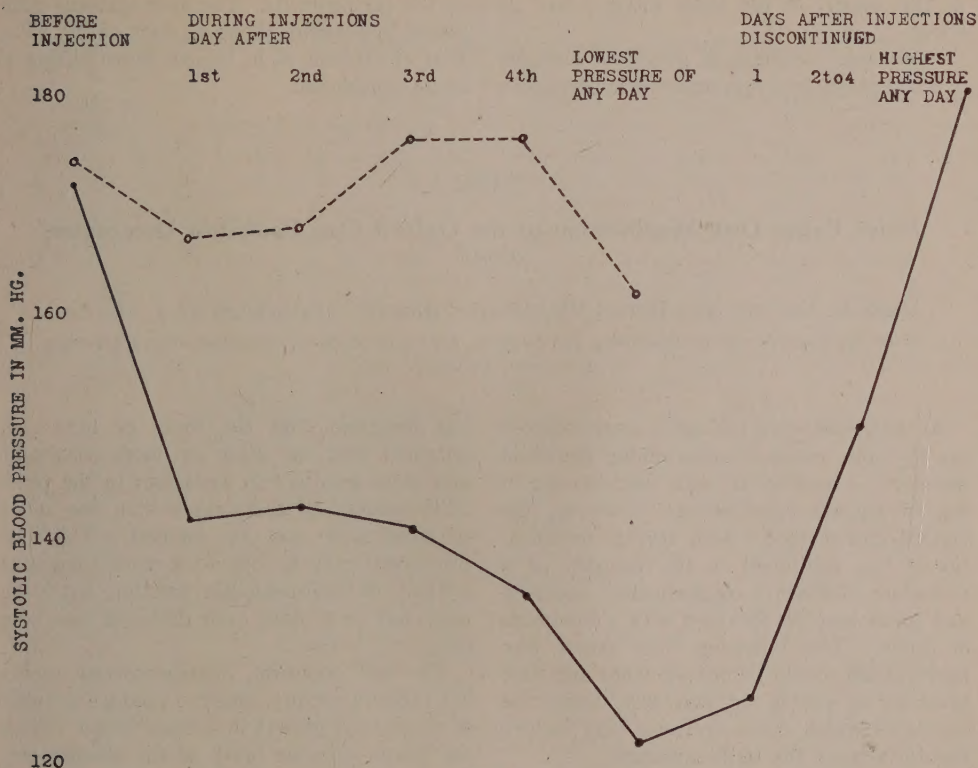


FIG. 1.

The solid line shows the average of 9 experiments before, during, and after 5 to 10 mg of Menadione daily. Another hypertensive animal did not respond to Menadione injections. The dotted line shows the average blood pressure figures of 5 rats before and during administration of 10 mg of Synkayvite daily.

blood pressure of experimental hypertensive rats after quinones of the vitamin K group had been administered.

Methods. The rats were rendered hypertensive by wrapping of both kidneys with silk. The blood pressure was determined by the platysmographic method of Grollman, Harrison and Williams.⁶

The substances used were 2-methyl-1,4-naphthoquinone, Menadione (Merck) which was dissolved in oil and given intramuscularly, and the water soluble hydroquinone compound 2-methyl-1,4-naphthohydroquinone, diphosphoric acid ester tetrasodium salt, Synkayvite (Roche), which was also applied by intramuscular injection.

Results. The results of the experiments are shown in the accompanying figure, from which it is evident that vitamin K (Menadione, Merck) is capable of lowering the blood pressure of hypertensive rats in a way similar to other quinones and to kidney extract. The hydroquinone compound (Synkayvite, Roche) showed no pressure-reducing activity. For any trial of treatment of human hypertension with vitamin K it must be borne in mind that much higher doses might have to be administered than those used for treatment of vitamin K deficiencies, since the doses found to be effective in hypertensive rats are relatively high.

That the result obtained with the Menadione is not due to the oil is shown by the fact that several other quinones, not reported

⁶ Grollman, A., Harrison, T. R., and Williams, J. R., *J. Clin. Invest.*, 1939, **18**, 373.

in this paper, in the same solvent, had no effect.

Summary. Vitamin K produces lowering of blood pressure in rats rendered hypertensive

by silk perinephritis. The hydroquinone compound Synkayvite shows no such effect. The trial of vitamin K in human hypertension is being considered.

14502

Filter Paper Disc Modification of the Oxford Cup Penicillin Determination.*

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At the present time biological assay methods are the sole means of determining penicillin potency. A number of such methods are in use in various laboratories;¹ however, the Oxford cup method,² with several modifications,³ has continued to be regarded as a procedure in which a commensurate accuracy and speed may be obtained with a minimum of labor. The following filter paper disc modification should reduce the labor and time involved in setting up this test, while the ease with which extra replicates may be run should increase the test's accuracy.

The principal modification lies in the use of a thick filter paper disc saturated with the penicillin sample, substituted for the sample-containing small cylinder used in the Oxford cup method. These discs may be conveniently set up on the seeded plates at a rate of about 6 per minute, a rate considerably more rapid than that in the original method. Another advantage of the discs is that the test plates may be manipulated freely to facilitate reading. Experience with a large number of tests

has indicated that the zones of inhibition obtained with the discs are more consistent and more sensitive to variations in the penicillin content of the sample than the zones obtained with the cup method. This improvement may be due to a more consistent contact of the penicillin solution with the agar and to a more even diffusion from the disc.

The test organism, *Staphylococcus aureus* H[†] (Oxford strain), tends to produce a somewhat granular growth in nutrient broth. With the use of peptone broth as the seeding medium, a more diffuse growth is obtained and this in turn effects a more even seeding of the test plates.

Hobby⁴ has criticized the plate method as having disadvantages arising from discrepancies in the depth and dryness of the agar and in the lag phase of the test organism. An attempt was made to control these factors by the use of a stabilized culture adapted to the seeding medium, and through a more complete standardization of the preparation and the treatment of the seeded plates.

Aside from the above modifications, the following procedure conforms closely to that of the Oxford method.

Procedure. The test organism, *Staphylococcus aureus* H, was transferred from an agar slant twice through peptone broth[‡] for 24-hour

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Rochester School of Medicine and Dentistry.

¹ Foster, J. W., and Woodruff, H. B., *J. Bact.*, 1943, **46**, 187.

² Abraham, E. P., Chain, E., Fletcher, C. M., Florey, H. W., Gardner, A. D., Heatley, N. G., and Jennings, M. A., *Lancet*, 1941, **2**, 177.

³ Foster, J. W., and Woodruff, H. B., *J. Biol. Chem.*, 1943, **148**, 723.

[†] Culture furnished through the courtesy of Dr. J. W. Foster, Merck & Co., Rahway, N.J.

⁴ Hobby, G. L., Meyer, K., and Chaffee, E., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50**, 277.

[‡] 1% peptone, 0.5% NaCl.

TABLE I.
 Zone Formation with Standard Penicillin.

Units in solution	Diameter of zones in mm	Avg zone size in mm	Units penicillin/ml		
			Mean value	Stand. dev.	Prob. error
4	32 32 32 31 32 33 32 32 32	32.1 $\pm$.335	4.058	1.072	$\pm$.258
2	30 30 29 30 30 31 29 30 30	29.9 $\pm$.3	2.01	0.347	$\pm$.083
1.5	28 28 28 29 29 29 29 29 30	28.8 $\pm$.15	1.418	0.306	$\pm$.073
1.0	28 28 28 27 28 28 28 28 28	27.9 $\pm$.074	1.02	0.084	$\pm$.02
.75	25 27 27 27 27 27 26 27 27	26.7 $\pm$.16	.726	0.104	$\pm$.025
.5	25 25 25 24 25 26 26 26 26	25.3 $\pm$.15	.521	0.071	$\pm$.017
.25	20 20 21 20 21 21 21 22 23	21.0 $\pm$.226	.249	0.038	$\pm$.009
.125	15 16 18 16 18 18 18 18 18	17.2 $\pm$.272	.121	0.028	$\pm$.007

growth periods at 37°C. The second transfer was held at 5°C for 16 to 18 hours.

Ten ml fresh nutrient agar was pipetted into uniform, flat-bottomed petri dishes and incubated for 16 to 18 hours at 37°C. These plates were held in the refrigerator for at least 1 hour.

Each plate was flooded with 1 ml of refrigerated culture. The excess was removed with a capillary pipette and the plates dried for 1 hour at 37°C (using wooden racks which supported the top half of the petri dish above the bottom half so that there was about ½-inch clearance). The plates were stored in the refrigerator, inverted, at least 1 hour.

Filter paper discs⁹ were sterilized by dry heat and immersed in the test fluid until saturated (30 seconds). They were then removed from the fluid with sterile forceps and, after gently shaking off the excess, were placed on the seeded plates. Special care should be taken to set the discs in place without smearing the surface of the agar. Three filter papers, evenly spaced, may be placed on each plate. For greater accuracy, plates may be run in triplicate. One of the discs on each plate may be a standard penicillin control.

The plates were incubated, not inverted, at 37°C for 14 hours (placed on wooden block to avoid excess condensation).

With a diffuse light a clear, well-defined zone of inhibition will appear around each disc if the test fluid contains more than 1/16 units of penicillin. By comparing the diameters of the zones to a standard curve of a

known sample, the concentration of penicillin may be determined.

Results. Table I shows an example of the results obtained by the disc method on a standard sample of penicillin.¹¹ This sample, treated turbidimetrically with a photoelectric colorimeter against growth in nutrient broth, produced complete inhibition of *Staphylococcus aureus* H at a concentration of just 0.02 units of penicillin per ml.

An examination of the data indicates the disc modification has materially improved the $\pm 25\%$ accuracy usually claimed for the Oxford method. The aforementioned results are quite typical of the probable error obtained in a considerable number of tests on commercial samples of penicillin. The sample should be diluted to approximately 1 unit per ml for the best results. For example, the above sample, containing 77 units per mg, showed a probable error of ± 1.54 units per mg where a 1 unit per ml dilution was used and ± 5 units per mg with a 4 unit dilution. Other investigators working with the Oxford method also have found that penicillin samples in the range of 1 unit per ml give the most exact readings.^{1,2,5}

The disc method has been found to work equally well on blood serum, spinal fluid, and urine. As in the original Oxford method, no filtration of the sample is necessary under ordinary circumstances.

Penicillin samples containing more than

¹¹ Penicillin standard furnished by Merek & Co., Rahway, N.J.

⁵ Atkinson, N., *Aus. J. Exp. Biol. and Med. Sc.*, 1943, **21**, 127.

⁹ No. 470, ½ inch diameter, Schleicher and Schuell Company, Inc., New York.

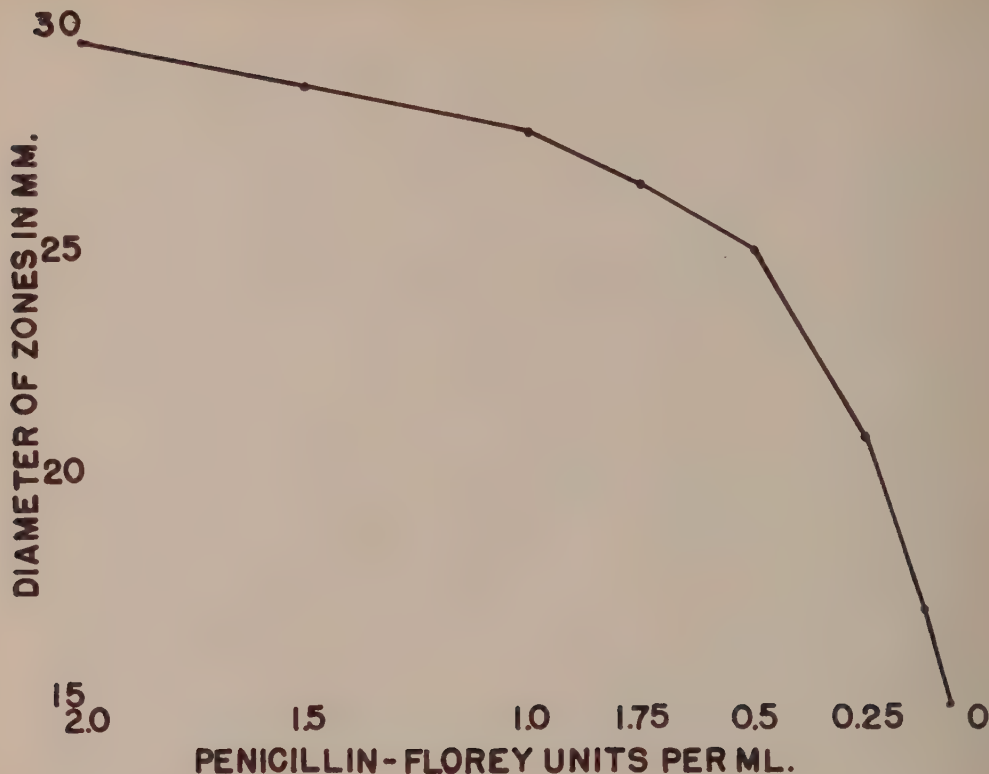


FIG. 1.

4 units per ml can be measured with the disc method, but with no great accuracy. Those containing as little as 1/16 units per ml produce a slight zone around the disc (diameter approximately 15 mm). Solutions of lower concentration cannot be measured by this method, but may be measured by the tube dilution method⁶ or its modification.⁷

Fig. 1 shows a standard curve obtained when the diameters of the zones of inhibition are plotted against the concentration of penicillin in Florey units.

⁶ Foster, J. W., *J. Biol. Chem.*, 1942, **144**, 285.

⁷ Rammelkamp, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 95.

Recognition of Liver Cirrhosis Induced by Paradimethylaminoazobenzene (Butter Yellow) by X-ray and Thorium Dioxide.

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A method of determining in the living albino rat the presence, the degree and the progression of liver damage induced by feeding butter yellow is presented. Changes of the spleen can also be observed. The advantages of the procedure are as follows: it permits continuation of an experiment until a desired lesion is obtained and it allows regeneration studies by establishing the severity of the lesion in given animals.

The procedure consists in administering intracardially 0.5 cc of thorium dioxide sol (thorotrast). A similar dose is given every 2 to 3 months if the experiment is prolonged over a long period. The thorotrast is deposited principally in the sinusoids of the liver and capillaries of the spleen. A roentgenogram allows the visualization of both of these organs. A satisfactory visualization is obtained by the use of low voltage, high amperage, and a fast exposure of 1/20 of a second. The rats can be put to sleep with ether or sodium pentobarbital if difficulties are experienced with the animal awake.

We observed in the haematoxylin and eosin preparations of cirrhotic liver tissue of rats fed butter yellow that the thorotrast was concentrated in the connective tissue. The thorotrast was intracellular. There was relatively little of the chemical in the sinusoids of areas in which liver cells were preserved. The thorotrast was singularly absent or only in minute quantities in parts of liver showing duct hyperplasia, duct adenocarcinoma, hepatoma, and adenocarcinoma of liver cells.

Correlation of the microscopic preparations and the roentgenograms indicated that the visible radiopaque lines outlined connective tissue which replaced liver cells and distorted the organ architecture. The nonvisualized areas represented the more or less intact liver tissue. Evaluation of the visualized and non-visualized area established the degree of damage and scarring.

Thorium dioxide sol is lodged largely in the capillaries of splenic pulp. There are occasional small accumulations of the chemical in the periphery of the follicles. Visualization of the spleen allows determination of its size. Spreading out of the radiopaque lines may be interpreted to indicate abnormal accumulation of substances in the pulp.

Intravenous administration of thorium dioxide in people to visualize the liver and the spleen was introduced by Radt¹ and Oka.² The method was used extensively for a number of years in the diagnosis of hepatic disorders until questions were raised of the possible injurious action of thorium dioxide. Stewart, Einhorn, and Illick³ demonstrated by shadowgrams the radio-activity of the chemical within the tissues. Orr, Popoff, Rosedale, and Stephenson,⁴ on the basis of their experiments, advised against administering thorium dioxide to human beings because the substance is not eliminated from the body, it blocks the reticuloendothelial system, it is radio-active and severely damages liver tissue. The last assertion is contrary to the findings of other investigators.⁵ Our own experience indicates that the tissue changes are minimal. A more recent report by Yater and Coe⁶ who followed 286 patients over a period of more than 10 years, denies any remote or immediate ill effects and stresses the value of the method in the diagnosis of cirrhosis, new growths, and abscesses of liver.

Haam, Tripoli, and Lehman⁷ employed

¹ Radt, P., *Klin. Wchnschr.*, 1929, **8**, 2128.

² Oka, M., *Fortschr. a. d. Geb. d. Röntgenstrahlen*, 1929, **40**, 497.

³ Stewart, W. H., Einhorn, M., and Illick, H. E., *Am. J. Roentgenol. and Rad. Therap.*, 1932, **27**, 53.

⁴ Orr, C. R., Popoff, G. D., Rosedale, R. S., and Stephenson, B. R., *Radiology*, 1938, **30**, 370.

⁵ Tripoli, C. J., *Am. J. Clin. Path.*, 1934, **4**, 212.

⁶ Yater, W. M., and Coe, F. O., *Ann. Int. Med.*, 1943, **18**, 350.

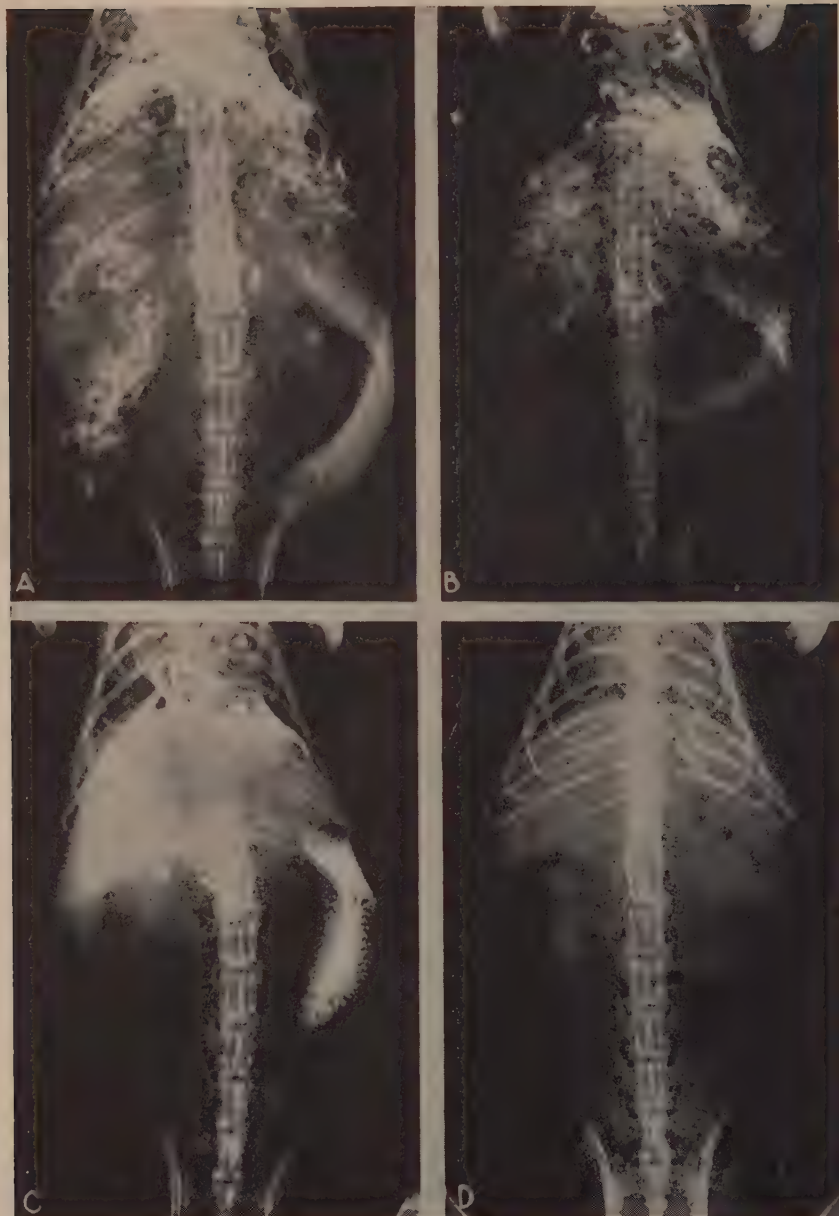


FIG. 1.

Roentgenograms of albino rats which were fed butter yellow and some of which were injected intracardially with thorium dioxide (thorotrast). A and B represent rats with different degrees of liver cirrhosis and splenic enlargement following a diet of ground brown rice, carrots, and butter yellow for approximately two months. Note the irregular outlines of liver tissue. The visualized lines correspond to connective tissue. Compare with C which is a visualization of a normal liver and spleen. D. Roentgenogram of an albino rat without thorium dioxide. The animal had liver cirrhosis corresponding in severity to that of B. Note absence of signs of liver disease.

thorium dioxide in studying contractions of the spleen in various animals. Tripoli⁵ obtained visualization of liver and spleen in dogs, rats, guinea pigs, mice, and rabbits, and Shute and Davis⁸ in rabbits and dogs. The latter investigators state that thorium dioxide prac-

tically disappears from the liver and spleen in 2 or 3 months and the cells regain almost a normal appearance.

Summary. A method of following the course of liver cirrhosis and splenic enlargement induced by feeding butter yellow to albino rats is described. The procedure consists of intracardiac injection of 0.5 cc of thorium dioxide sol (thorotrast) followed by roentgenograms of the liver and spleen.

⁷ Haam, V. E., Tripoli, C. J., and Lehman, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1932, **29**, 1056.

⁸ Shute, E., and Davis, M. E., *Arch. Path.*, 1933, **15**, 27.

14504

Potent Typing Sera Produced by Treatment of Donors with Isolated Blood Group Specific Substances.

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A rise in titer of isoagglutinins has been observed in patients following the erroneous transfusion of incompatible blood. There has been no uniform confirmation of this observation as in some instances the antibody titer did not change. Aubert, Boorman, and Dodd¹ reported an increase in isoagglutinin titer of A patients following the injection of several hundred cc of B plasma and of B patients treated with A plasma.[†] It would seem to be of interest to find out whether the purified group specific substances also will produce a rise in isoantibody titer when injected into human beings.

The blood group specific substances^{2,3,4,5}

* Observations made before entrance into active military service while fellow in Clinical Pathology, Buffalo General Hospital, Buffalo, New York.

¹ Aubert, E. F., Boorman, K. E., and Dodd, B. E., *J. Path. and Bact.*, 1942, **54**, 89.

[†] According to personal communication Wiener obtained increases in isoagglutinin titers in at least half of his donors following injection of pooled plasma.

² Witebsky, E., and Klendshoj, N. C., *J. Exp. Med.*, 1940, **72**, 663.

³ Witebsky, E., Klendshoj, N. C., and Swanson, P., *J. A. M. A.*, 1942, **116**, 2654.

⁴ Witebsky, E., Klendshoj, N. C., and Swanson,

used in the investigations to be reported were prepared by the Eli Lilly Company. The material consisted of a mixture of AB substance (isolated from the horse stomach) and A substance (isolated from the hog stomach.) Three to 5 mg AB substance and 5 to 10 mg A substance were dissolved in 10 cc of normal saline solution and dispensed in vials after being tested for sterility and potency.

The determination of the titer of the test sera was done in the following way: decreasing amounts of the donor's serum in volume of 0.2 cc were mixed with 0.2 cc of 1 to 2% suspension of washed, freshly-obtained blood cells belonging to different groups. After standing for 15 minutes, the tubes were centrifuged at medium speed for one minute. Reading of the degree of agglutination was done macroscopically after the tubes had been shaken slightly.

In the first series of experiments 3 donors belonging to groups O, A, and B received 10

P., *Neutralization of Isoagglutinins Anti-A and Anti-B in O Blood by Means of the Addition of the Isolated Blood Group Specific Substances, Blood Substitutes and Blood Transfusion*, Charles C. Thomas, 1942, 327.

⁵ Klendshoj, N. C., McNeil, Crichton, Swanson, P., and Witebsky, E., *Arch. Int. Med.*, 1942, **70**, 1.

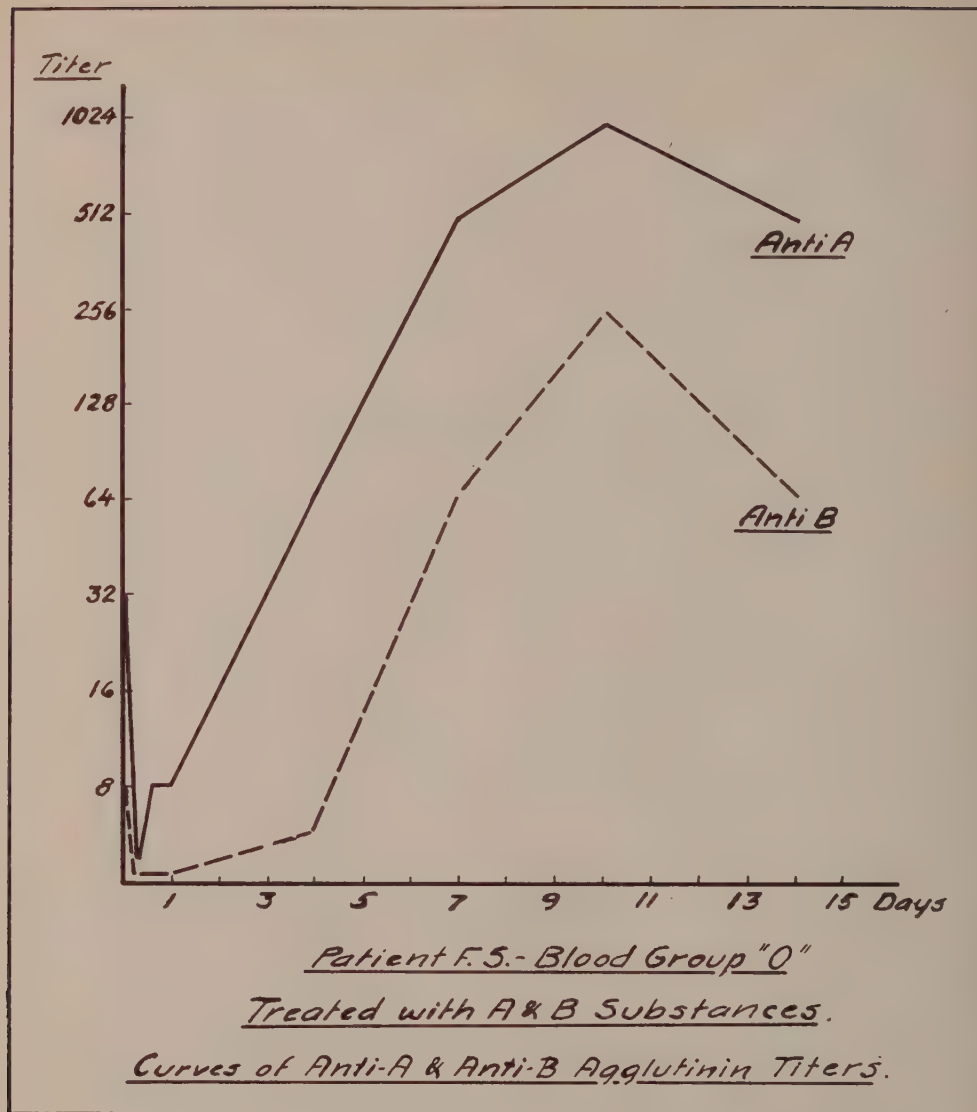


FIG. 1.

cc, or one vial, of the group specific substances, diluted in 100 cc saline solution, by the drip method. Fig. 1 illustrates the change in isoagglutinin titer of the donor belonging to group O following intravenous treatment with A & B substances.

The isoagglutinin anti-A was reduced by the injection of the material from a titer of 32 to 2, the isoagglutinin anti-B from a titer

of 8 to 1. After a few days the original titer was restored and the peak was reached 10 to 12 days following injection. A slight decrease in titer occurred during the observation period of two months. Yet after that period the titer was still far above the original.

The isoantibody titer of the second donor (Group B) rose only from 1:16 to 1:64 but the character of agglutination in higher serum

TABLE I.
Agglutination of B Cells by Serum of Patient
"G," Group A, Treated with A and B Specific
Substances.

Serum dilutions	Before treatment	After* treatment
1. 1:10	++++	++++
2. 1:20	+++	++++
3. 1:40	+	++++
4. 1:80	±	++++
5. 1:160	—	+++
6. 1:320	—	++
7. 1:640	—	+
8. 1:1280	—	+
9. 1:2560	—	+
10. 1:5120	—	±
11. 1:10,240	—	—
12. 0	—	—

* 17 days after termination of treatment.

Symbols: — = no agglutination; ± = very slight agglutination; + = slight agglutination; ++ = moderate agglutination; +++ = strong agglutination; and ++++ = very strong agglutination.

concentrations was much stronger in the serum specimen obtained after treatment as compared with that obtained before treatment.

The third donor belonging to blood group A experienced a rise in isoantibody anti-B from 8 to 256.

In the second series of experiments the treatment was changed and 4 consecutive injections, consisting of 0.1, 1, 3, and 10 cc, were given intravenously. The 0.1 cc used

for the first injection was taken up in 1 cc saline solution; the other injections were given with undiluted material.

Table I contains a comparison of serum titrations of a donor belonging to group A before and after intravenous treatment with 4 injections of A and B specific substances. An increase from 80 to a titer of about 5000 was observed in this case.

Subjected to the same treatment one donor did not show any considerable increase in antibody titer. Therefore, it was felt that too much of the A and B substances might have been injected considering the fact that one vial of the A and B substances proved to be potent enough to reduce the antibody titer present in the circulation of donors to the extent shown in Fig. 1. Another donor belonging to blood group O was given only one intravenous injection consisting of 0.1 cc of the stock A and B solution taken up in 1 cc pyrogen-free saline solution. The increase in antibody titer following the intravenous treatment with this small amount of A and B substances is shown in Table II.

The increase in antibody titer following the injection of minute amounts of A and B specific substances (0.03 to 0.1 mg of the purified A and B substances) is considerable. The isoantibody anti-A rose from a titer of

TABLE II.
Serum of Patient "R," Treated with Minute Amounts of A and B Substances.

Dilutions Serum "R," Group "O"	Before treatment		After treatment	
	A cells	B cells	Agglutination of A cells	B cells
1. 1:4	++++	+++	++++	++++
2. 1:8	+++	++	++++	++++
3. 1:16	++	+	++++	++++
4. 1:32	+	±	++++	++++
5. 1:64	±	—	++++	++++
6. 1:128	—	—	++++	++++
7. 1:256	—	—	++++	++++
8. 1:512	—	—	+++	+++
9. 1:1024	—	—	+++	++
10. 1:2048	—	—	+++	+
11. 1:4096	—	—	++	+
12. 1:8192	—	—	+	±
13. 1:16,384	—	—	±	—
14. 1:33,768	—	—	—	—
15. 1:67,536	—	—	—	—
16. 1:134,072	—	—	—	—
17. 1:268,144	—	—	—	—
18. 1:536,288	—	—	—	—
19. 1:1,072,576	—	—	—	—
20. 0	—	—	—	—

64 to about 16,000, the anti-B from 32 to 8000. Further investigations on a larger scale are necessary in order to determine the conditions which will produce maximum rise in antibody titer of donors treated with purified A and B substances. The experiment recorded in Table II suggests that minute amounts of A and B substances are able to stimulate a powerful antibody response.

All the injections described in this report have been given intravenously. To what extent subcutaneous, intradermal, or intramuscular injections can replace the intravenous administration is under investigation.[‡]

The important points regarding quality of a test serum are titer, speed, and specificity. The sera of donors treated with A and B substances excel by their speedy agglutination and produce complete agglutination of cells within a short time on slides and in test tubes. Even A₂ cells which are characterized by a relatively weak agglutinability are usually

strongly agglutinated in a short time. We have had no opportunity so far to examine A₃ cells. The specificity of the test sera obtained by treatment with A and B substances was in all instances satisfactory.

Conclusions. The intravenous treatment of donors with purified A and B specific substances isolated from animal sources leads to a considerable increase in isoantibody titer. The substances used were highly purified and did not contain any demonstrable hog or horse protein. The administration of even small amounts is effective and results in the production of sera which are characterized by high titer, speed and specificity. The procedure described easily permits the production of large quantities of very potent typing serum.

[‡] In one recent case of Group O the intramuscular injection of 1 cc of a stock solution of AB specific substances resulted in a 100-fold increase in both isoagglutinins Anti-A and Anti-B.

14505

Chemotherapy of Infections with *Cl. perfringens* in Mice by Homosulfonamides.*

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The homosulfonamides^{1,2} are not inhibited by *p*-amino benzoic acid.³ Disappointing results with the sulfonamides in the treatment of clinical gas gangrene⁴ due, in part at least, as far as their local use is concerned, possibly to the *p*-amino benzoic acid present in wounds, led us to investigate the toxicity and the efficacy of the homosulfonamides in experimental gas gangrene in mice. Reports on the use of homosulfanilamide (*p*-(α -amino)-toluene sulfonamide hydrochloride) have been inconclusive, owing partly to the variety of technics and the small numbers of animals used to test this compound.^{5,6,7,8,9} It has

been shown that homosulfanilamide was bacteriostatic *in vitro* for *Cl. perfringens* and for

¹ Miller, E., Sprague, J. M., Kissinger, L. W., and McBurney, L. F., *J. Am. Chem. Soc.*, 1940, **62**, 2099.

² Klarer, J., *Klin. Woch.*, 1941, **20**, 1250.

³ Schreus, H. T., *Klin. Woch.*, 1942, **21**, 671.

⁴ MacLennan, J. D., *Lancet*, 1943, **265**, 123.

⁵ Schreus, H. T., and Peltzer, E., *Klin. Woch.*, 1941, **20**, 504.

⁶ Schreus, H. T., Brauns, A., and Schümmer, H., *Klin. Woch.*, 1941, **20**, 1233.

⁷ Schreus, H. T., *Klin. Woch.*, 1942, **21**, 14.

⁸ Domagk, G., *Klin. Woch.*, 1942, **21**, 448.

⁹ Klöse, F., and Schröber, W., *Zent. f. Bakt.*, I Abt. orig., 1942, **149**, 15.

* Homosulfanilamide has been described in the German literature under the name of Marfanil.

TABLE I.
 Acute Toxicity of Homosulfonamides.

Drug	Solution injected		Dosage, g/kg	mM/kg	No. of mice	% mortality	Calculated LD ₅₀ and S.E. of LD ₅₀ as mM/kg
	%	pH					
132			3.0	13.5	25	0	18.5 ± 0.5
	8.0	4.3	4.0	18.0	45	42	
	10.0	4.5	5.0	22.5	25	88	
684	2.0	5.0	1.0	4.2	5	0	10.6*
	5.0	5.1	2.5	10.6	20	50	
			3.0	12.7	5	80	
			4.0	16.9	5	100	
627			1.0	3.3	5	0	4.37 ± 0.03
	2.4	4.0	1.2	3.9	20	25	
	2.8	4.1	1.4	4.6	20	65	
	3.2	3.9	1.6	5.2	20	90	
	4.0	3.9	2.0	6.6	5	100	
			3.0	9.9	5	100	
617			1.34	4.6	10	90	< 4.6
	3.08	5.6	1.54	5.2	10	90	
			1.92	6.6	5	100	
690	12.86	5.8	6.43	22.5	20	5	>22.5

* Approximation.

a strain of sulfathiazole-fast *Cl. perfringens*,¹⁰ and that bacteriostatic activity was not inhibited by *p*-amino benzoic acid.

The homosulfonamides, prepared in the Division of Medicinal Chemistry of the Squibb Institute,¹¹ were:

- 132—*p*-(α -amino)-toluene sulfonamide hydrochloride (homosulfanilamide)
 617—*p*-[N-(4,6 dimethyl) pyrimidyl-2]-(α -amino)-toluene sulfonamide (homosulfamethazine)
 627—*p*-(N-thiazyl-2) (α -amino)-toluene sulfonamide hydrochloride (homosulfathiazole)
 654—*p*-(N-thiazyl-2)-(α -phthalimido)-toluene sulfonamide
 684—*p*-(N-methyl) (α -amino)-toluene sulfonamide hydrochloride
 690—*p*-(α -succinamido)-toluene sulfonamide
 713—*p*-(α -sulfanilamido)-toluene sulfonamide

Toxicity Studies. The work was hampered by the lack of a satisfactory method of quantitatively determining these drugs. A comparison of the acute and chronic toxic effects

of 132 with its succinamido and several of its N substituted derivatives was made. (Sufficient amounts of 654 and 713 were not available. A mortality of approximately 60% was caused in mice receiving acacia suspensions of 654 or 713 intraperitoneally in doses of 0.6 or 2.0 g per kg respectively.) The compounds were used as soluble hydrochlorides, except the succinamido derivative which was dissolved in dilute alkali. Swiss mice were injected intraperitoneally and observed for 72 hours. The acute toxicity experiments are summarized in Table I. With a few exceptions, all deaths occurred within 2 hours after administration; none were observed after 24 hours. On the basis of millimols of drug per kilogram of body weight, all the derivatives, with the exception of the succinamido, were distinctly more toxic than 132.

The effects of continued administration of 132 and sulfanilamide were studied in groups of 10 mice each. The mice were put on diets containing 1, 2, and 4% of the former as well as equivalent levels (0.775, 1.55, and 3.1%) of the latter for a period of 28 days. The weight curves of the 7 groups (Fig. 1) demonstrate little or no difference in chronic toxicity of the 2 drugs at the 2 lowest concentrations. However, at the highest level

¹⁰ McKee, C. M., Hamre, D. M., and Rake, G., *Proc. Soc. Exp. Biol. and Med.*, 1943, **54**, 211.

¹¹ Braker, W., and Bergeim, F. H., to be published.

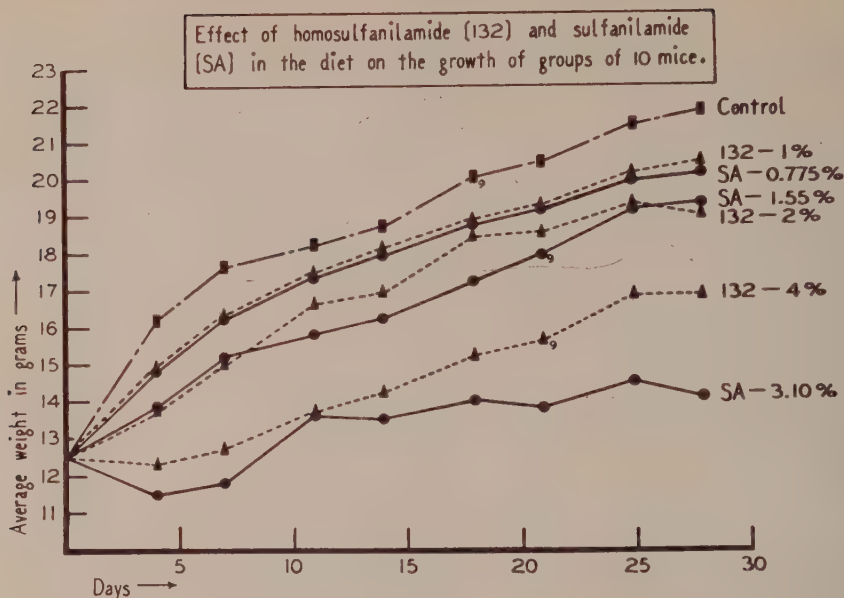


FIG. I

sulfanilamide had a distinctly greater effect than 132 on the growth of mice. One control mouse died on the 18th day of experiment, while on the 21st day one mouse each died on diets of 4% of 132 and 1.55% of sulfanilamide. On the 28th day the animals were sacrificed and the gross appearance of the organs of all mice was examined. The spleens of all mice receiving sulfanilamide were congested and slightly enlarged. Except for one mouse on the diet of 4% of 132, in which the spleen and kidneys were pale, no other gross changes were observed. Portions of the liver, kidneys, and spleen, of 4 mice on each diet were fixed for microscopic study. The only significant change in the livers of mice receiving 132 was a slight degree of fatty infiltration occurring on the highest drug level. All the mice receiving sulfanilamide were found to have hyperplastic spleens with large deposits of hemosiderin which also was unusually prominent in sections of the livers. One mouse on 4% homosulfanilamide had a marked hydronephrosis apparently owing to drug obstruction; a similar change, much less pronounced, was found in another mouse of this group. Tubular dilatation, apparently owing to mechanical obstruction, was also

observed in the kidneys of 2 mice on the highest diet level of sulfanilamide.

Therapeutic Studies. Mice were treated immediately after intramuscular infection with *Cl. perfringens*¹⁰ by injecting into the same site 0.1 ml of solutions of the homosulfonamides or sulfadiazine. They were observed for 3 weeks. It should be pointed out that this technic does not give entirely consistent results in repeated tests. In Table II are given all the results for compounds at the dose levels used in 3 or more tests. 654 and 713 were relatively insoluble, which in part accounts for the inconsistent results at the two dose levels of 713. In tests in which local treatment was delayed 1 hour, 132 still gave some protection.

At the dose levels employed, administered either in the diet or intraperitoneally (654—0.5% in diet, 684—500 mg/kg per day, 713—280 mg/kg per day or 0.14% in diet), 654, 684, and 713 did not protect mice infected intraperitoneally with *H. influenzae* 62/B, *Strep. hemolyticus* C203, or *Staph. aureus* Smith. When given by intraperitoneal injection (500 mg/kg per day), 132 did not protect mice infected intraperitoneally with *Strep. hemolyticus* or *Staph. aureus*, while at

TABLE II.
Local Treatment of Mice Injected Intramuscularly with *Cl. perfringens*.

Drug	Mg inj.	No. of tests	No. of mice	No. lived	No. died	% lived
NaSD*	1.0	5	40	37	3	92
"	0.1	7	60	52	8	87
132	1.0	3	30	23	7	77
"	0.5	6	60	35	25	58
617	1.0	3	25	10	15	40
627	1.0	3	25	9	16	36
654†	1.0	3	30	12	18	40
684	1.0	4	40	28	12	70
"	0.5	5	50	32	18	64
690	1.0	3	30	10	20	33
713†	1.0	4	40	2	38	5
"†	0.5	3	30	15	15	50
Controls	—	17	150	6	144	4

* Sodium sulfadiazine.

† Suspended in acacia.

1% in the diet, a slight degree of protection against *Strep. hemolyticus*, but not against *Staph. aureus*, was obtained. 132 at 1% in the diet did not protect mice infected with *Cl. perfringens*, although 1% sulfathiazole protected 50%. That these results were not due to non-absorption of 132 was shown by detection of this drug in the urine by means of *in vitro* tests with *Cl. perfringens*.

Three of the compounds were tested for bacteriostatic action on 15 strains of staphylococci by a serial dilution test in charcoal-adsorbed beef heart infusion broth, containing very little *p*-amino benzoic acid.¹² At 6.2 mg % all the strains were inhibited by 132 and 684 for 10 hours, although some grew in 17 hours. 132 and 684 also prevented growth of all strains for 17 hours when present in a concentration of 25 mg %, although by 57

hours all strains had grown. 690 had no demonstrable action.

Summary. Except for sulfadiazine, homosulfanilamide and its N¹-methyl derivative were the most effective drugs in local treatment of experimental gas gangrene of the compounds tested. The acute toxicity of homosulfanilamide was less than that of its derivative. The chronic toxicity of homosulfanilamide in mice appeared to be no greater than that of sulfanilamide. These two compounds were not effective in the treatment of infections in mice with *Strep. hemolyticus* C203, *H. influenzae* 62/B, or *Staph. aureus* Smith, but they were bacteriostatic *in vitro* for staphylococci. Although infection with *Cl. perfringens* in mice is not strictly comparable with infection in man, these results indicate that homosulfanilamide may be worthy of a clinical trial in the local treatment of gas gangrene.

14506

Differentiation Between Fievre Boutonneuse and Rocky Mountain Spotted Fever by Means of Complement Fixation.

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It has been shown by Badger¹ that Fievre

Boutonneuse, a disease of the Mediterranean basin transmitted by *Rhipicephalus sanguineus* ticks, and Rocky Mountain spotted fever are related, for one disease protects

¹ Badger, L. F., *Public Health Rep.*, 1933, **48**, 507.

against the other in cross immunity tests in guinea pigs. On the other hand, Davis and Parker² have shown that a vaccine prepared from inactivated Rocky Mountain spotted fever rickettsiae (tick vaccine) which protects against Rocky Mountain spotted fever affords no protection against Fievre Boutonneuse. While the reverse has not been demonstrated, namely, whether a vaccine prepared from inactivated Fievre Boutonneuse rickettsiae confers an immunity to Rocky Mountain spotted fever, the work referred to indicates that while there is a group relationship between these diseases, there are some antigenic differences.

Because of the cross immunity conferred in guinea pigs by these two diseases laboratory differentiation may be difficult. The following communication presents data to indicate that these two diseases can be differentiated by means of specific complement fixation.

The antigen for the complement fixation test in Rocky Mountain spotted fever is best prepared from tissue cultures on agar.³ This antigen is specific and with it Rocky Mountain spotted fever can be differentiated from either epidemic or murine typhus fever. This method is used routinely in the Division of Virus and Rickettsial Diseases at the Army Medical School for the diagnosis of Rocky Mountain spotted fever.

The antigen for Fievre Boutonneuse is prepared in the identical manner as those described for epidemic and murine typhus.⁴ The antigen is prepared from yolk sac cultures from fertile hens' eggs. The infected yolk sacs are harvested and inactivated with 0.5% formalin. The rickettsiae are extracted and further purified by centrifugation in the cold in an angle centrifuge at 4,000 r.p.m. for one hour and washed 4 times in buffered saline, pH 7.0. In this instance, as with the typhus antigens, it was found that a soluble antigen is liberated in the supernatant fluid. The soluble antigen obtained from typhus rickettsiae gives cross fixation with epidemic and

murine convalescent serum while the soluble antigen obtained from Fievre Boutonneuse cultures gives cross fixation with Rocky Mountain spotted fever convalescent serum. (Protocol III). A soluble antigen is also found in agar tissue cultures of Rocky Mountain spotted fever.⁵ In both instances however the washed rickettsial bodies provide a specific antigen.

The method of performing the tests has been previously described.^{3,4}

Protocol I presents the results obtained with convalescent guinea pig serum from animals recovered from Fievre Boutonneuse, Rocky Mountain spotted fever, epidemic and murine typhus using purified Fievre Boutonneuse and Rocky Mountain spotted fever rickettsial antigens.

This experiment demonstrates that specific complement fixation can be obtained with guinea pig convalescent Fievre Boutonneuse or Rocky Mountain spotted fever serum when the homologous antigen is used but no fixation is obtained with the heterologous antigens. Convalescent epidemic typhus, murine typhus, and normal guinea pig serum gives no fixation with either of these antigens.

Protocol II shows the results obtained with human Fievre Boutonneuse and Rocky Mountain spotted fever convalescent serum using purified Fievre Boutonneuse and Rocky Mountain spotted fever rickettsial antigens.

All specimens were negative using an epidemic or murine typhus antigen. Likewise, 5 human epidemic typhus and 5 murine typhus convalescent specimens were negative using a Fievre Boutonneuse or Rocky Mountain spotted fever antigen.

The results indicate that with Fievre Boutonneuse convalescent serum there is some cross fixation in low titer, but a higher titer is obtained with the homologous antigen. It is of interest to note that the highest titer occurred in a specimen of serum obtained from the most recent case (1 year) but a significant titer persisted 10 years after an attack of Fievre Boutonneuse.

With Rocky Mountain spotted fever convalescent serum some cross fixation is obtained

² Davis, G., and Parker, R. R., *Pub. Health Rep.*, 1934, **40**, 423.

³ Plotz, H., and Wertman, K., *Science*, 1942, **95**, 441.

⁴ Plotz, H., *Science*, 1943, **97**, 20.

⁵ Unpublished data.

PROTOCOL I.
Reactions with Convalescent Guinea Pig Serum (Bled 2 weeks after infection).

Disease	Guinea pig No.	Serum titer with F.B. antigen*	Serum titer with R.M.S.F. antigen*
Fievre Boutonneuse	1889	1/20	0
	1890	1/20	0
	1891	1/80	0
	1895	1/160	0
	1897	1/80	0
	1898	1/80	0
Rocky Mountain spotted fever	Pool A	0	1/320
	" B	0	1/80
	" C	0	1/80
Epidemic typhus	4 pools; C.F. positive for epidemic typhus	0,0,0,0	0,0,0,0
Murine typhus	4 pools; C.F. positive for murine typhus	0,0,0,0	0,0,0,0

* Only 4 and 3 plus reactions read as end-point titers.

PROTOCOL II.
Reactions with Human Fievre Boutonneuse and Rocky Mountain Spotted Fever Convalescent Serum.

Disease	Case No.	Time after onset	Serum titer with F.B. antigen*	Serum titer with R.M.S.F. antigen*
F.B.	1	1 yr	1/320	1/20
	2	2 "	1/40	0
	3	5 "	1/40	0
	4	6 "	1/80	1/10
	5	10 "	1/20	0
R.M.S.F.	1	140 days	1/40	1/640
	2	31 "	1/160	1/640
	3	217 "	0	1/80
	4	14 "	0	1/160
	5	29 "	0	1/80
	6	146 "	0	1/80
	7	30 "	1/10	1/1280
	8	57 " (1st spec.)	1/10	1/320
		120 " (2nd ")	1/20	1/640
	9	5 " (1st ")	0	0
		64 " (2nd ")	1/20	1/320
	10	75 " (1st ")	1/10	1/320
		155 " (2nd ")	1/20	1/640

* Initial dilution of serum.

with a Fievre Boutonneuse antigen thus demonstrating antigenic relationship. In all cases, however, when cross fixation does occur in Rocky Mountain spotted fever the titer obtained with a Rocky Mountain spotted fever antigen was higher than was obtained with the Fievre Boutonneuse antigen. In those cases where specimens were obtained at two periods during the course of the disease a rise in antibody titer was observed with the homologous antigen, but no significant rise occurred with the Fievre Boutonneuse antigen.

As was previously pointed out the soluble antigen obtained from washings of Fievre

Boutonneuse yolk sac cultures gives cross fixation with Rocky Mountain spotted fever convalescent guinea pig serum. Protocol III demonstrates this point. The same Rocky Mountain spotted fever pooled serum which gave fixation with the washings obtained from the Fievre Boutonneuse cultures gave negative results when subsequently tested with the purified Fievre Boutonneuse rickettsial antigen.

Summary. Data are presented to indicate that Fievre Boutonneuse can be differentiated from Rocky Mountain spotted fever by means of complement fixation provided purified

PROTOCOL III.
 Fievre Boutonneuse Supernatant (4,000 R.P.M.)

Antigen dil.*	1/10	1/20	1/40	1/80	1/160	1/320	1/640	Serum control 1/10
	Serum Dilutions—Fievre Boutonneuse Convalescent G.P. Pool.							
1/3	4	4	4	4	3	2	1	0
1/4	4	3	2	1	0	0	0	
1/6	1	0	0	0	0	0	0	
1/8	0	0	0	0	0	0	0	
	Serum Dilutions—Rocky Mountain Spotted Fever G.P. Pool.							
1/3	4	4	4	4	4	4	4	0
1/4	4	4	4	4	3	3	2	
1/6	4	3	1	0	0	0	0	
1/8	2	1	0	0	0	0	0	
	Serum Dilutions—Normal G.P. Pool.							
1/3	2	1	0	0	0	0	0	0
1/4	0	0	0	0	0	0	0	
1/6	0	0	0	0	0	0	0	
1/8	0	0	0	0	0	0	0	

* Antigen anticomplementary at 1/2 dilution.

ricketsial antigens are used. This test may be employed with convalescent guinea pig or human serum thus providing a convenient laboratory test for diagnosing Fievre Boutonneuse. Since there is some cross fixation between Fievre Boutonneuse and Rocky Mountain spotted fever further evidence is presented to indicate that these diseases are antigenically related. Other members of the Spotted Fever Group are now being similarly

studied.

The authors wish to thank Dr. R. R. Parker, Rocky Mountain Laboratory, for his kindness in giving us the Fievre Boutonneuse strain, Captain T. E. Woodward for obtaining the Fievre Boutonneuse human convalescent sera, Captain B. L. Bennett and Lt. A. B. Scoville, Jr., for maintaining the strain in guinea pigs and collecting the specimens of sera, and Corporal A. Rudolph for his technical assistance.

14507 P

Development of Tumors in the Rat Ovary After Transplantation into the Spleen.

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It has been shown by Golden and Sevringhaus¹ that rats in which the ovaries have been transplanted to the mesentery remain anestrus, owing to inactivation in the liver (previously reported by Zondek²) of the estrogen thus secreted into the portal circulation.

This phenomenon was more extensively studied by G. R. Biskind³⁻⁶ using pellets of

crystalline steroids implanted in another organ in the portal circulation, the spleen. Castrate

¹ Golden, June B., and Sevringhaus, E. L., *PROC. SOC. EXP. BIOL. AND MED.*, 1938, **39**, 361.

² Zondek, B., *Skandinav. Arch. f. Physiol.*, 1934, **70**, 133.

³ Biskind, G. R., and Mark, J., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 212.

⁴ Biskind, G. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1940, **43**, 259.

⁵ Biskind, G. R., *Endocrinology*, 1941, **28**, 218.

⁶ Biskind, G. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1941, **46**, 452.

* Aided by grants from the Winthrop Chemical Company, the S.M.A. Corporation, and the United Hospital Fund of New York.

rats on a complete diet with pellets of estrone or estradiol thus implanted likewise remained anestrus. During the course of an investigation on the effects of nutritional deficiency on the estrogen-inactivating function of the liver,⁷⁻⁹ M. S. Biskind and Shelesnyak¹⁰ demonstrated that animals with one ovary removed and the other transplanted to the spleen showed no estrual reactions so long as the diet was adequate to maintain the functional integrity of the liver.

This observation seemed to provide an excellent means of ascertaining the effect on



FIG. 1.

(S43-1089. 100 $\times$ approximately). Small nest of granulosa cells situated in theca cell mass.

⁷ Biskind, M. S., and Biskind, G. R., *Science*, 1941, **94**, 462.

⁸ Biskind, M. S., and Biskind, G. R., *Endocrinology*, 1942, **31**, 109.

⁹ Biskind, M. S., and Biskind, G. R., *Endocrinology*, 1943, **32**, 97.

¹⁰ Biskind, M. S., and Shelesnyak, M. C., *Endocrinology*, 1942, **30**, 819.

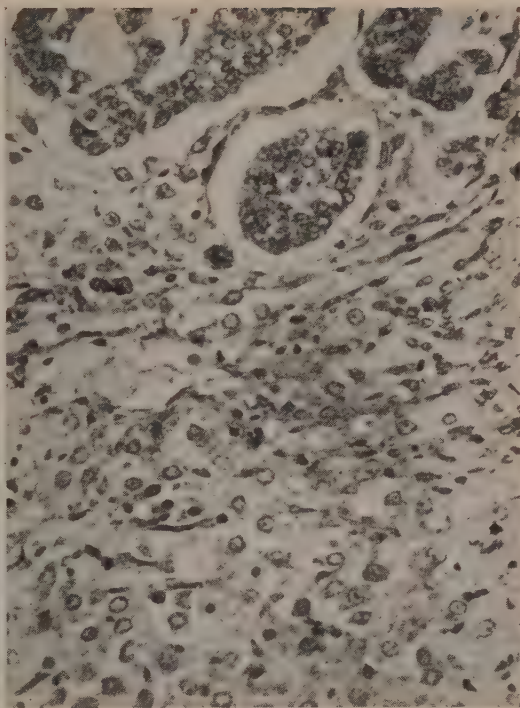


FIG. 2.

(S43-1089. 200 $\times$ approximately.) Granulosa cells in ball-like clusters and forming small cysts; some theca cells are luteinized.

the ovary of protracted stimulation by the pituitary, under conditions in which the latter organ is freed from the normal inhibitory action of the secreted estrogen.

Accordingly, a series of adult female rats of the Sherman strain were castrated and one ovary was transplanted to the spleen.[†] After an interval of approximately 2 weeks, all the animals had daily vaginal smears to rule out those with vascularized adhesions that permitted ovarian estrogen to by-pass the portal circulation. Those showing estrous reactions were discarded. The others were maintained on a complete diet[§] thereafter for approximately 11 months. At the end of this time

[†] We are indebted to Dr. M. C. Shelesnyak for his assistance in performing the ovarian-splenic transplantations.

[§] Rockland pellet diet with frequent supplements of lettuce, carrots, and meat.

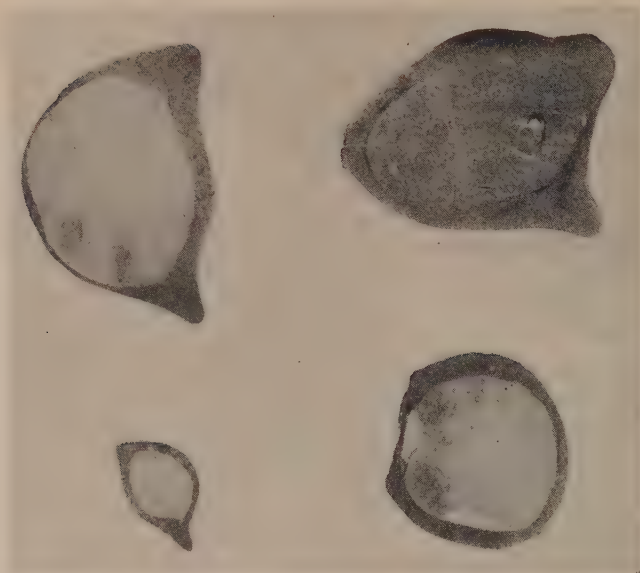


FIG. 3.

Three of the larger ovarian transplants described in the text, compared with a transplant of normal size¹⁰ (lower left) all photographed to the same scale, approximately 3 $\times$. Figs. 1 and 2 are from the specimen in the lower right.

9 animals remained; they were again smeared daily over a period of 3 weeks to determine whether any estrogen was reaching the systemic circulation. Six remained completely anestrus; 3 had smears showing the occasional presence of a few epithelial or squamous cells but none of the latter at any time showed a complete estrous smear.

The animals were killed and the spleen containing the transplanted ovary, the vagina and uterus and the pituitary were preserved for histologic examination. In every case the ovarian mass in the spleen was much enlarged, the smallest measuring 7 x 5 x 3 mm, and the largest 15 x 13 x 10 mm. Normal ovaries removed from unoperated rats of the same strain and same age measured not larger than 5 x 4 x 3 mm.

On histologic examination all the ovarian transplants were found to be greatly hypertrophied and composed of masses of theca cells; all follicles had completely disappeared. In 3 of the transplants large tumors were found, apparently of the granulosa cell type,

and in 2 others smaller nodules of the same cellular structure were found. With but 2 exceptions, the uterus and vagina showed castrate atrophy and the pituitaries were also of the castrate type. Two of the animals showed a slight estrogenic effect on the vagina, virtually none on the endometrium, and the pituitaries were of a modified castrate type. Thus for the most part, it appears that none of the estrogen secreted by the transplanted ovaries, or very little of it, was able to escape inactivation in the liver and the secretory activity of the pituitary was therefore entirely or largely unopposed by the inhibitory action of estrogen.

The granulosa cell tumors, as shown in the accompanying illustrations, are fairly sharply demarcated from the theca cell masses. They are made up of anastomosing cords and plugs and solid ball-like masses of small cells, indistinctly outlined. These cells have scant cytoplasm and a small round nucleus filled with fine chromatin granules. Mitotic figures are numerous in some regions, as many as 3 to 5

in each high-dry field. In some areas the cells are arranged to form small cystic cavities containing a colloid-like substance, as in the "folliculoid" type of tumor. The vascular network is not prominent and there is very little discernible fibrous stroma. In many respects these resemble the ovarian tumors

found by Brambell¹¹ and by Furth¹² in the ovaries of mice following roentgen irradiation.

¹¹ Brambell, F. W. R., Parkes, A. S., and Fielding, U., *Proc. Roy. Soc., Lond., Ser. B*, 1937, **101**, 29.

¹² Furth, J., and Butterworth, J. S., *Am. J. Cancer*, 1936, **28**, 66.

14508

Development of Resistance to Pyrithiamine in Yeast and Some Observations on Its Nature.

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Since the development of resistance to various specific antimicrobial agents is a problem of much practical as well as theoretical interest, it has seemed advisable to attempt the production of variants of susceptible microorganisms which would be insusceptible or fast to pyrithiamine, and to study the means by which this fastness was achieved. It has recently been shown that pyrithiamine, the pyridine analog of thiamine, was a highly selective and effective inhibitor of the growth of microorganisms¹ and animals.² Only those organisms which were caused to grow more abundantly by the presence of thiamine were inhibited in growth by pyrithiamine. It was also shown¹ that the insusceptible species did not owe their resistance to the production of thiamine or other antagonist of pyrithiamine. If a fast strain of a susceptible species could be developed, it would be of interest to learn what alteration, if any, would occur in its thiamine requirements.

A pyrithiamine-fast strain of *Endomyces vernalis* was obtained in the following manner. Twelve successive transfers of the strain previously used were made in the synthetic medium,¹ to which had been added graded amounts of pyrithiamine ranging from 2 to 20

μg per cc. Consecutive transfers were made, beginning with the lowest concentration and terminating at the highest. After the yeast had been treated in this manner, its susceptibility to pyrithiamine was the same as that of the parent strain. It was then transferred 30 times in the synthetic medium plus 25 μg of pyrithiamine per cc. Care was taken to make each successive transplant as soon as growth was visible. Although in the first transfers growth appeared very slowly, after about the twentieth turbidity developed in 8 hours of incubation at 37°. The final culture obtained was labeled PF *Endomyces*.

PF *Endomyces* grew as well in a medium containing 25 μg of pyrithiamine per cc as it did in the absence of the compound. This was 25 times the amount of pyrithiamine which was sufficient to inhibit the growth of the parent strain half-maximally. However, the PF strain grew more slowly in the absence of pyrithiamine than did the parent strain. With the same size of inoculum, the PF strain required 40 hours to produce the same amount of growth made by the parent strain in 24 hours.

The PF strain had the same thiamine requirements as the parent strain. Thus it grew very poorly in the absence of thiamine, and was stimulated to grow either by thiamine or by its pyrimidine portion[†] alone. The quanti-

* With the technical assistance of J. Backstrom and J. Clifford.

¹ Woolley, D. W., and White, A. G. C., *J. Exp. Med.*, 1943, **78**, 489.

² Woolley, D. W., and White, A. G. C., *J. Biol. Chem.*, 1943, **149**, 285.

[†] Throughout this paper, the pyrimidine portion of thiamine refers to 2-methyl-4-amino-5-hydroxymethyl-pyrimidine.

TABLE I.
Effect of Thiamine and of Pyrithiamine on Growth
of PF *Endomyces*.
Colorimeter readings represent % of incident light
transmitted by the cultures.

Thiamine μg per cc	Pyrithiamine μg per cc	Colorimeter reading
0	0	72
0.01	20.0	54
0.01	0	61
0.003	0	69
0.001	0	70
0	0.5	54
0	0.1	54
0	0.05	56
0	0.025	60
0	0.01	67
0	0.005	69

tative requirements for these substances were the same as for the parent strain. The PF strain was thus the first organism observed which was insensitive to pyrithiamine and yet was stimulated by thiamine. Some data to illustrate these points are shown in Table I.

In the absence of thiamine, pyrithiamine was a growth factor for PF *Endomyces* (Table I). On a molecular basis, pyrithiamine was 50% as active as thiamine in promoting growth.

The fastness of the PF strain to pyrithiamine, as well as its ability to use the inhibitor in place of the vitamin, was partially explained by the observations that pyrithiamine was destroyed, and that the pyrimidine portion of thiamine was produced from pyrithiamine during growth. Apparently the inhibitor was cleaved between the rings by a system which had arisen in the PF strain. To demonstrate this, PF *Endomyces* was grown for 40 hours at 37° in the synthetic medium plus 1 μg of pyrithiamine per cc. The cells were removed and the culture filtrate was assayed for thiamine (or its pyrimidine half) according to the method described previously.¹ It was found to contain 0.5 μg of thiamine or its pyrimidine half per cc. This value agreed well with the finding that pyrithiamine had 50% of the growth-promoting potency of thiamine for the PF strain. When the PF strain was grown in the presence of 20 μg of pyrithiamine per cc, the percentage conversion to free pyrimidine was 39%.

Although the pyrimidine portion of thiamine had some effect in reversing the action of pyrithiamine on *Endomyces vernalis*,¹ it was less than 1% as active as thiamine in this respect. Since thiamine and its pyrimidine portion were equally effective as a growth factor, it was possible to determine thiamine in the presence of its pyrimidine portion by adding pyrithiamine to the basal medium. The growth effect observed under this condition was due almost entirely to the intact vitamin. By use of such a procedure, the active material in the culture filtrates was indicated to be the pyrimidine rather than thiamine.

Destruction of pyrithiamine was not the sole means by which the PF strain protected itself against the inhibitor, for when the filtrates obtained from cultures which contained originally 20 μg of pyrithiamine per cc were assayed for pyrithiamine with the parent strain, 20% of the inhibitor was found still to be present. This was sufficient to cause inhibition of growth of the parent strain and yet not to have an effect on the growth of the PF strain. The inhibition of growth of the parent strain was overcome by thiamine.

The production of the pyrimidine portion of thiamine from pyrithiamine was also accomplished by species naturally resistant to the inhibitor. When *Escherichia coli* was grown in a synthetic medium plus 1 μg of pyrithiamine per cc, pyrimidine activity equivalent to 47% yield from the pyrithiamine was found in the culture filtrate.

Summary. A strain of *Endomyces vernalis* was obtained by growing the yeast in media containing pyrithiamine. This strain was not affected in growth by concentrations of pyrithiamine 25 times greater than that required to inhibit the growth of the parent strain half-maximally. The new strain still required thiamine or its pyrimidine portion as a growth factor. In the absence of thiamine, pyrithiamine was 50% as active as the vitamin for growth of the variant. When grown in small concentrations of pyrithiamine and in the absence of thiamine, the new strain converted half of the pyrithiamine to the pyrimidine portion of thiamine.

Absence in Lymph of Capillary Permeability Factors in Traumatic Shock.

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It has been postulated by various investigators¹ that in traumatic shock, substances increasing capillary permeability are liberated in the injured tissues. It might be expected that such substances appear in the lymph draining the traumatized region in higher concentration than in blood. In an attempt to demonstrate such factors, canine lymph was collected during shock and tested in rabbits for its effects on capillary permeability.

Shock was produced by vigorous intestinal massage, peripheral circulatory collapse being verified by blood pressure, hematocrit and autopsy observations. Lymph was obtained from a duct lying between the first and second intestinal nodes, prior to massage, and shortly before death during the period of permanently depressed blood pressure (below 70 mm Hg.). Simultaneously femoral vein blood was drawn for plasma and serum samples. Permeability effects were determined in rabbits according to Menkin's method² (intradermal injection of 0.1 cc samples of test fluids, followed by intravenous injection of 2.0 cc per kg of a 1% saline solution of Trypan Blue).

In an initial series of 12 dogs and 15 rabbits, in which heparin was used as anticoagulant, the skin reactions did not show any significant difference between the responses to control and to "shock" fluids: a slight to a marked increase in permeability occurred with each

type of fluid. Heparin controls were negative.

In a second series of 12 dogs and 6 rabbits, in which defibrinated fluids were tested without heparin, again no differences were observed between reactions to control and to shock samples; in these instances the responses were only slight or negative. Similarly, both control and shock fluids caused practically the same degree of extravasation of dye in a third series of experiments using 8 dogs and 8 rabbits, in which the non-protein fractions of lymph and of plasma were compared after the proteins had been removed by acetone precipitation; all reactions again were much weaker than in the first series.

Freeman and Schechter observed recently³ that canine lymph from traumatized limbs had no effect on capillary permeability when re-injected into the skin of the same animals. These investigators obtained the test fluids within a period of 3 hours after traumatization. In the experiments reported here the "shock" samples of lymph and blood were collected only after peripheral circulatory failure had been produced, on an average approximately 9 hours after traumatization. Not only whole lymph and plasma, but also the defibrinated fluids and their non-protein fractions were tested in rabbits for effects on capillary permeability. Even under these conditions, we were unable to demonstrate substances, formed as a consequence of traumatic shock, which increase capillary permeability.

* Present address: Institute of Experimental Biology, University of California, Berkeley.

¹ Recently reviewed in Moon, V. H., *Shock*, 1942, Lea and Febiger, Philadelphia.

² Menken, V., *J. Exp. Med.*, 1936, **64**, 484.

³ Freeman, N. E., and Schechter, A. E., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 29.

Mixtures of Pure Amino Acids as Substitute for Dietary Protein.

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Rose¹ has stated that a diet containing 10 essential amino acids is adequate for growth. Animals on this diet gained weight just as rapidly as when protein components were supplied preformed. Evidence has recently been presented by Albanese and Irby² showing that a mixture of essential amino acids is inferior from a nutritional standpoint to enzymatic or acid casein hydrolysates or casein. They interpret their results as indicating the toxic effect of unnatural forms of certain amino acids.

The synthesis of vitamins in the intestinal tract of the rat having been amply demonstrated,³⁻⁹ an attempt to obtain evidence suggesting similar synthesis of amino acids was considered advisable.

Albino male rats of the Wistar Institute strain about 35 days old were placed on diets of the following composition: Enzymatic casein digest, vitamin free casein, or amino acid mixture, 16; sucrose, 69; salts, 4, cod liver oil, 2; corn oil, 9; NaHCO₃, 2. The vitamin supplements were added at the following levels per kilogram of diet: thiamine hydrochloride, 5 mg; riboflavin, 10 mg; pyri-

doxine, 5 mg; nicotinic acid, 100 mg; calcium pantothenate, 100 mg; choline chloride, 200 mg; inositol, 200 mg; *p*-amino benzoic acid, 100 mg; ascorbic acid, 100 mg; 2-methyl-1,4-naphthoquinone, 50 mg; ethyl linolate, 10 g; alpha-tocopherol, 100 mg; biotin (conc. No. 5000 SMA), 200 μ g. The amino acid mixture had the following composition: *l*-(+)-lysine HCl, 50.5 g; *dl*-valine, 45.0 g; *l*-leucine, 36.0 g; *dl*-isoleucine, 40.5 g; *dl*-threonine, 23.0 g; *l*-tryptophane, 9.0 g; *l*-histidine, 15.75 g; *dl*-phenylalanine, 33.75 g; *dl*-methionine, 31.5 g; and *l*-(+)-arginine, 13.5 g. To this diet there was added in some instances, succinyl-sulfathiazole at a 2% level replacing an equivalent amount of sucrose. Six sets of 5 rats each were placed on the various diets. Food consumptions of all the rats were measured in order to eliminate this factor in interpretations of the growth rates. As there was no variation, the values are not listed.

A consideration of the growth values indicates that in the series on Diets A, C, and D the greatest growth was obtained on the casein-containing diet, next on the enzymatic digest of casein, and least on the purified amino acids. The rats receiving purified amino acids doubled their weights in 7 weeks. This indicates that Rose¹ was partially correct in his assumption, and precludes to a certain extent the possibility of toxic action of the unnatural isomers, as in our diet A and the diet EAA of Albanese and Irby² the same unnatural amino acids occur. In the diet of du Vigneaud *et al.*,¹⁰ the unnatural isomer used is similar to that of Albanese and Irby.² The diet of Bauer and Berg¹¹ contains unnatural isomers in approximately the same amounts and yet they report moderate growth.

¹ Rose, W. C., *Physiol. Rev.*, 1938, **18**, 109.

² Albanese, A. A., and Irby, V., *Science*, 1943, **98**, 286.

³ Block, S., McKibbin, J. M., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1941, **47**, 308.

⁴ Martin, G. J., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 353.

⁵ Daft, F. S., Ashburn, L. L., and Sebrell, W. H., *Science*, 1942, **96**, 321.

⁶ Black, S., Overman, R. S., Elvehjem, C. A., and Link, K. P., *J. Biol. Chem.*, 1942, **145**, 137.

⁷ Lockhart, E. E., Sherman, H., and Harris, R. S., *Science*, 1942, **96**, 542.

⁸ Light, R. F., Cracas, L. J., Olcott, C. T., and Frey, C. N., *J. Nutr.*, 1942, **24**, 427.

⁹ Wakim, K. G., Krider, M. M., and Day, H. G., *Proc. Soc. Exp. Biol. and Med.*, 1943, **54**, 164.

¹⁰ du Vigneaud, Vincent, Dyer, H. M., and Kies, M. W., *J. Biol. Chem.*, 1939, **130**, 325.

¹¹ Bauer, C. D., and Berg, C. P., *J. Nutr.*, 1943, **26**, 51.

TABLE I.
 Weekly Weight Gain on Various Diets.

Diet	Initial wt, g	Weekly wt gain in g, avg for 5 rats							% dead end of 7 wks	Avg total gain 7 wks, g
		1	2	3	4	5	6	7		
A 16% Amino Acid Mixture	52.0	9.4	5.6	8.4	10.0	3.0	7.3	5.2	0	48.9
AS 16% Amino Acid Mixture + 2% Sulfasuxidine	50.0	1.2	1.6	3.1	—1.0	—3.0	—5.5	—1.0	100	—5.6
C 16% Casein	49.5	17.0	11.4	13.6	21.0	16.0	12.1	15.3	0	106.4
CS 16% Casein + 2% Sulfasuxidine	51.8	17.6	11.2	10.4	10.2	1.0	3.0	11.0	20	64.4
D 16% Enzymatic Casein Digest	42.0	14.0	18.6	12.4	10.0	8.5	7.2	15.5	20	86.2
DS 16% Enzymatic Casein Digest + 2% Sulfasuxidine	47.0	11.6	12.6	7.2	4.0	—10.0	—1.0	8.0	40	32.4

In the present work, the growth could be considered moderate, although not the equal of the rats on casein diet, and there was no evidence of toxicity or nutritive failure such as reported in the rats of Albanese and Irby.² One interesting point of difference lies in the somewhat higher content of *dl*-valine in the diet of Albanese and Irby.² The unnatural form of valine is not available to the rat. Leucine, isoleucine, lysine, and threonine are other amino acids for which a similar situation holds true but there is no significant difference in the diets in this respect, and one would not expect any toxicity to result from the inclusion in a diet of the unnatural amino acid isomer if that isomer is available.

Comparison of the growth rates of rats on Diet A and Diet AS shows clearly the detrimental effect of the inclusion of succinylsulfathiazole. This drug is not toxic at a level of 2% when included in 18% casein diets supplemented with folic acid concentrates. As is well known, the sulfa drug does precipitate many vitamin deficiencies. The findings here reported are suggestive of inhibition of amino acid synthesis by bacteria in the intestine of the rat. Even on a 16% casein diet plus 2% succinylsulfathiazole, the rats grow very well; this situation also obtains when the casein is replaced by an enzymatic digest of casein incorporated at the same level. The amounts of amino

acids available to us were not of sufficient magnitude to permit studies determining which of the other amino acids are essential in experiments of this type. Thus, it is our theory that as in the case of the vitamins so it is with the amino acids: certain members of each group are synthesized in the intestinal tract and thus do not appear to be essential; however, with the inclusion of slowly absorbed sulfonamides this synthesis is reduced and the deficiencies are precipitated.

Enough folic acid concentrate, prepared by the method of Hutchings *et al.*,¹² was available to feed 2 rats on each of the above sulfasuxidine-containing diets for 7 weeks at a level of 5 mg daily. The results indicate exactly the same conclusions as had been obtained with the diets lacking folic acid. Growth on Diet CS supplemented with folic acid nearly equalled that in rats on stock diets. The rats on the AS diet supplemented with folic acid did not gain weight and died in the sixth week. The rats on the diet DS grew but not so well as the rats on diet CS when both were supplemented by folic acid.

Summary. When succinylsulfathiazole is incorporated into a diet containing all known vitamins plus ten essential amino acids, inadequate nutrition is found to result.

¹² Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, 1941, **141**, 521.

The Antiriboflavin Effect of Isoriboflavin.*

GLADYS A. EMERSON AND MAX TISHLER.

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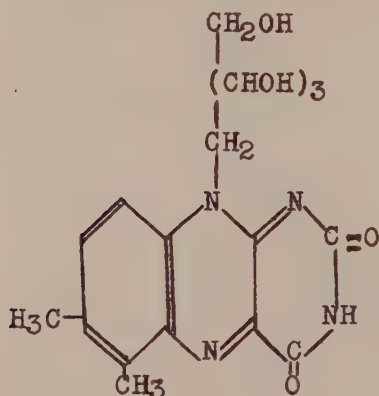
Several investigators have reported on the antagonism existing between certain B vitamins and structurally related compounds. Following the discovery of the antagonism between sulfanilamide and para-aminobenzoic acid by Woods,¹ pyriethamine,² pyridine-3-sulfonic acid,³ and pantooytaurine⁴ have been reported as inhibitors of thiamine, nicotinic acid and pantothenic acid respectively. Kuhn and co-workers⁵ have recently reported that 6,7-dichlor-9-d-riboflavin inhibits the growth of riboflavin requiring microorganisms.

An isomer of riboflavin, namely, 5,6-dimethyl-9-(*d*-1'-ribityl)-isoalloxazine (termed isoriboflavin) when assayed for riboflavin activity was found to suppress growth both in riboflavin-deficient rats and in rats receiving a sub-optimal intake of the vitamin.

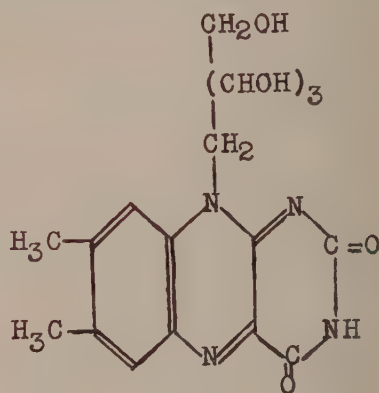
Forty-two male weanling rats were placed on the riboflavin-deficient ration and segregated into 6 like groups treated as follows:

1. No riboflavin.
2. No riboflavin and 2 mg of isoriboflavin daily.
3. Sub-optimal level of riboflavin (10 μ g daily).
4. Sub-optimal level of riboflavin (10 μ g daily) and 2 mg of isoriboflavin.
5. Optimal level of riboflavin (40 μ g daily).
6. Optimal level of riboflavin (40 μ g daily) and 2 mg of isoriboflavin.

The basal ration consisted of vitamin-free casein 18%; dextrose 68%; salt mix U.S.P. XI No. 1 4%; Crisco 8%; cod liver oil 2%; supplemented with 0.8 mg each of thiamine and pyridoxine, 5 mg of Ca pantothenate, 10



Isoriboflavin



Riboflavin

* We are indebted to Dr. Karl Pfister and Miss Eleanor Smart of Merck & Co., Inc., for the synthesis of this compound. The synthesis of this compound will be described elsewhere.

¹ Woods, D. D., *Brit. J. Exp. Pathol.*, 1940, **21**, 74.

² Woolley, D. W., and White, A. G. C., *J. Biol. Chem.*, 1943, **149**, 285.

³ McIlwain, H., *Brit. J. Exp. Pathol.*, 1940, **21**, 136.

⁴ Snell, E. E., *J. Biol. Chem.*, 1941, **139**, 975.

⁵ Kuhn, R., Weyand, F., and Möller, E. F., *Ber.*, 1943, **76**, 1044.

TABLE I.
Growth Response of Rats to Riboflavin and Iso-riboflavin (Both compounds were dosed as a suspension in 0.5 ml gum acacia).

No.	Group	Gain in wt 28 days (Avg initial wt, 46 g)	Gain in wt Succeeding 21 days
		Period I g	Period II* g
1.	Riboflavin deficient 0.5 ml gum acacia	15	
2.	2 mg Isoriboflavin	5	
3.	10 μ g riboflavin	69	94
4.	10 μ g riboflavin + 2 mg isoriboflavin	29	118
5.	40 μ g riboflavin	96	
6.	40 μ g riboflavin + 2 mg isoriboflavin	92	

* Riboflavin increased to 80 μ g daily between periods I and II.

mg of nicotinamide, and 100 mg of choline chloride per 100 g of diet.

The riboflavin and isoriboflavin were fed by stomach tube as a suspension in gum acacia.

Isoriboflavin inhibited the growth of rats in the absence of riboflavin (Group 2, Table I, Period I) although the manifestations of deficiency (greasiness of the fur and alopecia) and the survival time were not significantly different between the animals in groups 1 and 2. The analog, in the dosage employed, markedly depressed the growth of the rats receiving a sub-optimal intake of the vitamin (Groups 3 and 4). The growth-depressing effect of isoriboflavin was almost completely overcome by the feeding of 40 μ g of riboflavin (Groups 5 and 6).

Thus, it would appear that the effect of isoriboflavin was that of an inhibitor of ribo-

flavin.

The counteraction of large doses of the vitamin upon the inhibiting effect of the analog was further shown in the animals of Groups 3 and 4. After 28 days on test the level of riboflavin was increased from 10 μ g to 80 μ g for these groups: the feeding of isoriboflavin to the rats in group 4 was continued at the level of 2 mg. As evidenced by the growth response (Table I, Period II), as well as by the general improvement in the condition of the animals, the inhibiting effects of isoriboflavin were overcome by the curative feeding of 80 μ g of riboflavin.

Summary. The feeding of isoriboflavin to rats receiving a sub-optimal intake of riboflavin inhibited the growth-promoting action of the vitamin. The antagonistic effect of the isomer was prevented or overcome by the feeding of an adequate level of riboflavin.

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Influence of Bacteriologic Peptones on Hydrogen Sulfide, Indol and Acetyl Methyl Carbinol Production by Enterobacteriaceæ.

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The production of hydrogen sulfide (H₂S) by members of the family Enterobacteriaceæ is regarded by microbiologists as an important differential diagnostic criterion. In addition, the production of indol and acetyl methyl carbinol (A.M.C.) is used as a means of separating two significant genera, namely, the

Aerobacter from the Escherichia. Bacteriologic peptones serve as sources of (a) sulfur compounds for H₂S formation, (b) tryptophane for indol formation, and (c) compounds possessing the guanidine nucleus necessary for the detection of A.M.C. That peptones vary between manufacturers is almost axiomatic.

These differences may be due to the variations in chemical composition which in turn are determined by (a) the substrate from which the peptones are prepared and (b) methods of preparation. It occurred to the writers that the brand and kind of peptone might, therefore, materially influence any or all of the above three diagnostic reactions.

Peptones recommended as good sulfur sources for the H_2S test seem to be a matter of choice. Levine¹ *et al.*, Hunter and Crecelius,² Utermohlen and Georgi³ recommend Bacto proteose-peptone while ZoBell and Feltham⁴ found Bacto tryptone to be quite satisfactory; the Society of American Bacteriologists' "Manual of Methods"⁵ suggests any peptone containing available sulfur as being satisfactory. As for sources of tryptophane, Tilley⁶ found that peptones varied considerably in their content of this amino acid; he studied relatively few brands. Recently, however, Hook and Fabian,⁷ employing the Rosenheim test for tryptophane, obtained strong positive tests with all the commercial peptones investigated by them. The "Manual"⁵ recommends casein digests such as Bacto tryptone. As for the presence of compounds possessing the guanidine structure, Barritt⁸ found Bacto peptone to be satisfactory while the "Standard Methods"⁹ and the S.A.B. "Manual"⁵ demand the use of either Witte or Difco proteose peptone for the Voges-Proskauer test. It is

apparent, therefore, that certain commercial peptones are superior to others.

Not only has the sulfur source been an important factor in the production of H_2S , but the selection of a suitable detector ion is likewise essential. A number of compounds have been reported to yield satisfactory results. Lead, in the form of the acetate, is the detector ion used almost exclusively and is the accepted one in the technic outlined in the S.A.B. "Manual."⁵ Levine¹ successfully employed iron (ferric citrate) while ZoBell and Feltham⁴ and others² have found bismuth (citrate) to be suitable detectors of sulfide production. The latter two elements have been reported to be superior to lead because of their lower toxicity toward bacteria. What effect different peptones may have on either the toxicity of Pb^{++} , Fe^{+++} or Bi^{+++} or upon detection of sulfides by the latter, has never been extensively determined. That differences do exist were noted³ but were not thoroughly investigated. It is the purpose of this paper to determine those differences.

Materials. Twenty commercial bacteriologic peptones were employed. The names of the peptones and the manufacturers appear in Table I. Bacterial cultures employed for the H_2S tests were as follows (number in parentheses indicates number of strains used): *Aerobacter aerogenes* (2), *Aerobacter oxytoca* (1), *Eberthella typhosa* (2), *Proteus vulgaris* (2), *Salmonella typhimurium* (2), *Salmonella schottmuelleri* (2), *Salmonella pullorum* (2), *Shigella gallinarum* (2), *Shigella sonnei* (1), *Shigella dysenteriae* (2), *Escherichia freundii* (1), *Streptococcus faecalis* (1). For the indole and Voges-Proskauer tests, the following were used: *A. aerogenes* (7), *A. cloacae* (1), *A. oxytoca* (1), *E. coli* (7). The *Escherichia* and *Aerobacter* were found to be "typical" in their biochemical reactions.

Methods. Media used for the detection of H_2S was prepared in accordance with the procedure recommended by (a) the S.A.B. "Manual"⁵ with lead acetate, (b) Levine¹ with Fe as the indicator, and (c) Hunter *et al.*² with Bi as the detector. The only variable in each of these media was the pep-

¹ Levine, M., Vaughn, R., Epstein, S. S., and Anderson, D. O., *PROC. SOC. EXP. BIOL. AND MED.*, 1932, **29**, 1022.

² Hunter, C. A., and Crecelius, H. G., *J. Bact.*, 1938, **35**, 185.

³ Utermohlen, W. P., and Georgi, C. E., *J. Bact.*, 1940, **40**, 449.

⁴ ZoBell, C. E., and Feltham, C. B., *J. Bact.*, 1934, **28**, 169.

⁵ *Manual of Methods for Pure Culture Study of Bacteria*, Leaflet V, 1942, Society of American Bacteriologists, Geneva, N.Y.

⁶ Tilley, F. W., *Am. J. Pub. Health*, 1921, **11**, 834.

⁷ Hook, A. E., and Fabian, F. W., *Tech. Bull. No.* 185, 1943, Mich. Agr. Exp. Sta.

⁸ Barritt, M. M., *J. Path. Bact.*, 1936, **42**, 441.

⁹ *Standard Methods for the Examination of Water and Sewage*, 8th ed., 1936, Am. Pub. Health Assn., New York, N.Y.

TABLE I.

Comparison of Pb, Fe, and Bi as Indicators of H_2S Using 20 Peptones as Sulfur Sources and 20 Cultures Representative of the Enterobacteriaceæ.

Peptones			No. cultures yielding vigorous positive H_2S tests			No. cultures growing but failing to produce H_2S			No. cultures failing to grow in presence of following		
Name	Manufacturer	Code	Pb	Fe	Bi	Pb	Fe	Bi	Pb	Fe	Bi
Peptone	Pfanzstiehl	P	9	6	6	10	20	4	0	2	9
Nutri Peptone	Wilson	W	12	0	16	1	17	0	0	0	0
Bacteriologic Peptone	Parke-Davis	PD	14	9	7	0	6	1	2	0	6
Peptone Siccum	Stearns	S	8	0	11	6	20	1	0	0	4
"	Armour	AS	7	0	6	5	20	3	0	0	6
Peptic Digest Pork Muscle*	"	AP	9	3	16	8	9	0	0	0	4
Pancreatic Digest Beef Muscle*	"	AB	6	0	15	8	17	1	0	0	2
"	"	AC	9	0	15	2	20	0	1	0	1
Pancreatic Digest Casein	"	AI	0	0	18	20	19	0	0	0	0
Infusion Peptone	"	PL	19	6	11	0	10	1	0	0	5
Bacteriological Peptone	Paul-Lewis	PL	19	6	11	0	10	1	0	0	5
Hog Protein Peptone	Cudahy	CHP	0	0	3	3	20	5	14	0	11
Hog Peptone	"	CH	8	3	9	6	12	2	0	0	3
Beef Peptone	"	CB	12	5	14	3	6	4	2	0	1
Bacto Tryptose	Difco	DT	11	5	16	6	11	0	2	0	1
Proteose Peptone	"	DI	10	3	16	4	6	0	1	0	2
"	"	DP	11	0	9	2	16	3	5	1	5
Bacto Peptone	"	DY	2	0	17	15	20	0	0	0	0
Bacto Tryptone	"	DN	1	0	18	0	17	1	19	0	0
Neopeptone	"	DN	1	0	18	0	17	1	19	0	0
Proteose Peptone No. 3	"	DPP	8	8	11	9	11	7	2	0	0
+ $NaHSO_3$	"	DPP	8	8	11	9	11	7	2	0	0
Witte's Peptone	Fr. Witte	WT	0	3		0	16		20	0	

* Experimental peptones.

tone as the sulfur source.

The media used for the detection of indol and acetyl methyl carbinol production were both prepared as prescribed in the "Standard Methods."⁹ The only variable in each medium again was the peptone which in the first test served as a source of tryptophane and in the second as a source of a guanidine-containing compound. The test for indol was Kovac's reagent⁹ but A.M.C. was detected by the sensitive procedure outlined by Barritt.⁸ Tests for the latter were conducted at 24- and 72-hour intervals.

Results. A. Hydrogen sulfide. From the data in Table I, it is apparent that many more positive responses to H_2S production were obtained when Bi was employed as the indicator ion. Ten peptones in particular were found to possess components which, when converted to sulfides by bacterial reduction, produced darkening along the line of inoculation. The best were W, AP, AB, AC, AI, DT, DI, DY, and DN. The intensity of the test for peptone CB was high (Fig. 1) but only 14 of the 20 cultures gave satisfactory results. There were some cultures which

grew in the presence of Bi but failed to produce H_2S ; these were considerably fewer than when either Fe or Pb were used as the detector ion (Column 3, Table I). Peptone WT formed a dark precipitate with Bi on autoclaving, making that combination useless. In general, Bi seemed to produce an unmistakable positive test.

Hydrogen sulfide detection by ferric iron was generally poor. A great number of cultures grew in the presence of Fe but failed to produce H_2S . Best results were obtained with peptones PD and DPP. In the presence of peptone DI, 3 cultures were good sulfide producers while 11 failed to yield this gas. Peptones PL, CB, and DT showed tests of good intensity, in terms of iron (Fig. 1), however only 25% of the cultures responded vigorously. In general, the iron sulfides were the weakest in intensity.

With 7 peptones (W, PD, PL, CB, DT, DI, and DP), 50% or more of the cultures yielded strongly positive tests when Pb was the detector. About 3 times as many cultures grew in the presence of Pb and did not produce H_2S than in the case of Bi. The intensity

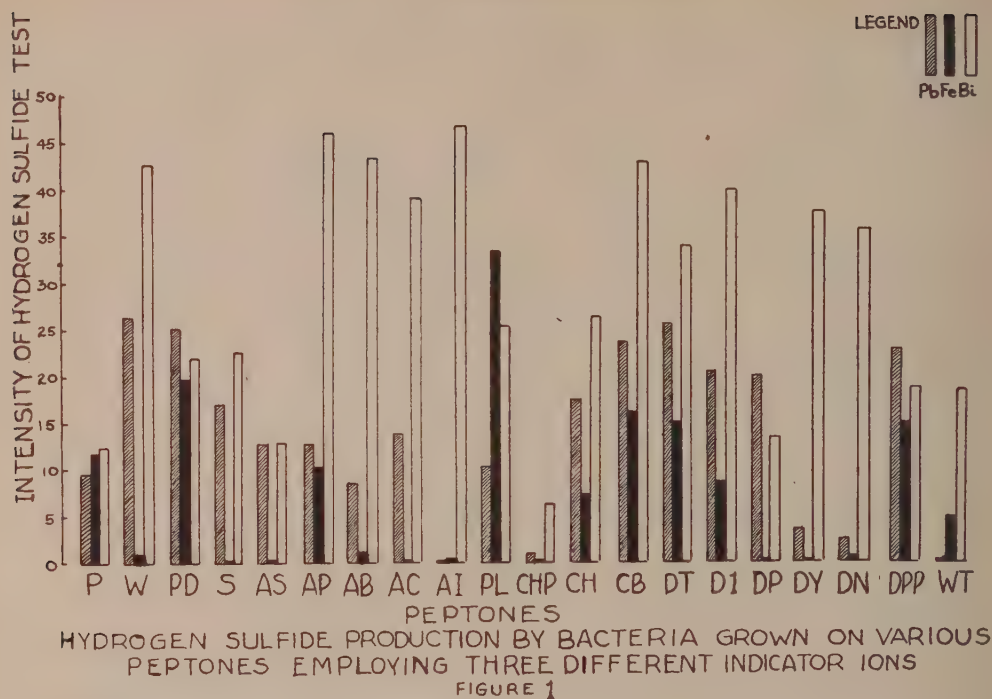


FIGURE 1

of the tests was not of the order of Bi but was superior to Fe. Peptones AI, DY, and DN are unusual in that very few positive tests were reported with Pb but many with Bi.

B. Growth. The growth of some bacterial cultures was completely inhibited by two of the elements depending upon the peptone used. Iron was toxic only 3 times in 400 trials. Lead and bismuth were about equal, *i.e.*, 68 per 400 and 60 per 380 trials respectively. Peptones CHP, DN, and WT accounted for 53 of the 68 lead inhibitions whereas with bismuth the distribution was quite even amongst the 20 sulfur sources.

C. Indol production. The results indicate that two peptones, S and CHP, failed completely to yield indol whereas one (P), is a questionable source of available tryptophane. Fourteen others were satisfactory and of these the most clear cut positive tests were produced in peptones DI, AS, AC, and WT, while W, PL, DT, and DY gave very good results based upon the intensity of the test and color of the reaction mixture.

Several of the peptones produced water-

white aqueous solutions which made the cherry-red color reaction appear more pronounced than where it was partially masked by a yellow tinge frequently encountered in solutions of peptones.

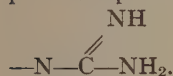
The Hopkins-Cole test for the detection of tryptophane was *positive* for 2 peptones (S, CHP) which failed to yield indol biologically. Obviously, the tryptophane is present but in combination with other constituents of the peptone which render it unavailable to the bacteria.

There is still another possible explanation as to the variation in the indol response, *i.e.*, the pH of the medium. Tilley⁶ found a direct relation between hydrogen ion concentration and indol production; peptones whose aqueous solutions were near neutrality yielded positive results. Of the 4 peptones which gave the most striking positive results, solutions of 3 gave pH's near neutrality and one (AS) was acid. Peptones DI, AC, and WT were then acidified to pH 5.4 and AS brought to neutrality; following inoculation, tests were again made for indol, the results being in exact

agreement with those obtained on the same peptones before neutralization or acidification. Of 4 other peptones, which also gave good tests, 3 were nearly neutral and the fourth was acid. Hydrogen ion concentrations of aqueous solutions of the peptones yielding either variable results or wholly negative tests, were between pH 5.2 and 5.9. Neutralization, before inoculation, failed to improve the indol test. Of the remaining 7 peptones which gave moderately good indol tests, 4 were nearly neutral and 3 were acid.

It appears that there is a slight correlation between the indol response and the "natural" pH of the aqueous solution of a peptone. However, alteration in hydrogen ion concentration of peptones normally yielding good indol tests or peptones yielding poor tests failed to bring about a reversal in response to Kovac's reagent.

D. Acetyl methyl carbinol production. In the Voges-Proskauer test, the A.M.C. condenses with constituents of the peptone containing the guanidine nucleus to give a red color in the presence of alkali. The peptone is of value then, in this test, because it supplies compounds having the configuration



From Barritt's¹⁰ investigations, it appears that the brand of peptone materially influences the speed with which the Voges-Proskauer test becomes readable. Some peptones permit the test to be detected in 24 to 48 hours while others can be read only at 72 hours. In our studies, tests which were positive or negative at 24 hours were the same at the end of 72 hours.

Seven peptones were found to be slightly superior to the remaining peptones used while 3 strains of *A. aerogenes* were variable in their activity. The 7 peptones are S, AS, AP,

AB, AC, CB, and DT. Peptone PL was unsatisfactory. Barritt¹⁰ found Witte's peptone (WT) to be wholly unsatisfactory.

No strains of *E. coli* were V-P positive. Hydrogen ion concentrations of the glucose-peptone waters after autoclaving ranged from pH 7.0 to 7.3.

Discussion. From the experimental data on hydrogen sulfide production, it becomes apparent that the ability of a peptone to render sulfur available to bacteria and to support their growth is correlated with the character of the indicator ion incorporated in the medium. The sensitivity of the detector ions to the H₂S test differs, viz., Bi > Pb > Fe. However, the order of toxicity is Pb > Bi > Fe. Perhaps the microbial enzyme which brings about the reduction of sulfur is inactivated in some bacteria to a greater extent by certain metallic ions than by others or possibly the indicator combines with the sulfur compound forming a complex rendering it unavailable.

The hydrogen ion concentration of aqueous solutions of peptones apparently influences the availability of tryptophane for indol production. Where the pH is favorable and indol is not produced by bacterial action, it is possible that the tryptophane may be chemically bound so as to be biologically unavailable although the Hopkins-Cole test was positive.

Compounds containing the guanidine nucleus, possibly arginine, must be abundantly distributed in commercial peptones for in no instance did this appear to be a limiting factor.

Summary. Hydrogen sulfide production by bacteria varied greatly with the indicator ion employed; bismuth was superior, as a detector, to iron and lead. Iron was the least toxic. Occasionally the tryptophane content but rarely the guanidine configuration serve as limiting factors in peptones used for differential bacterial tests.

¹⁰ Barritt, M. M., *J. Hyg.*, 1943, **43**, 214.

14513 P

Use of B-Proteus OX19 Agglutination in Diagnosis of Cancer.

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In a recent study the sera of pregnant women uniformly were found to contain a high titer of agglutinins for B-proteus OX19. In the course of that study¹ it appeared that the sera of women with malignant neoplastic disorders likewise strongly agglutinated proteus organisms. Even though the reaction was non-specific, yet it was believed that the agglutination test often might provide evidence for the existence of cancer.

For this reason the titer of B-proteus OX19 agglutinins* was measured^{2,3} in the sera of 30 normal women, 27 with mammary carcinoma, and 3 with carcinoma of the cervix. The diagnosis of carcinoma in each instance

was confirmed by microscopic examination of biopsied sections.

The results of this study failed to indicate any difference between the sera of the two groups (Table I). Of the 30 sera taken from persons having cancer, 14 gave positive agglutinations with B-proteus OX19, the titer ranging from 1:20 to 1:160 (only one serum agglutinated as high as 1:160). Of the control group, 21 sera showed positive reactions, the titers ranging from 1:20 to 1:80.

Conclusion. By the methods used, there was no evidence that the agglutination of B-proteus OX19 antigen would be of value in determining the presence of cancer.

TABLE I.
The Agglutination Titer of the Sera Studied for B-Proteus OX19.

	Titers				
	0	1:20	1:40	1:80	1:160
Control individuals	9	7	9	5	0
Patients with mammary or cervical carcinoma	16	6	5	2	1

¹ Gratch, I., *Am. J. Surg.*, 1943, **60**, 411.

* Lederle Laboratory antigen.

² Welch, H., *Am. J. Pub. Health*, 1937, **27**, 141

(Yearbook).

³ Welch, H., and Mickle, F. L., *Am. J. Pub.*

Health, 1936, **26**, 248.

14514

Effect of Cooling Isolated Parts upon the Comfort of Man Resting in Hot Humid Environment.*

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Hill¹ and Kuno and Ikeuchi² inhibited sweating in human subjects by cooling iso-

* Aided by a grant in aid from the Rockefeller Foundation.

¹ Hill, L., *J. Physiol.*, 1921, **54**, 137.

² Kuno, G., and Ikeuchi, K., *J. Orient. Med.*, 1928, **9**, 53.

lated portions of the body. They made no attempt to correlate the temperature of the bath cooling the part, the temperature and humidity of the environment, and the size of the area cooled, with the quantity of sweat and the comfort of the subjects. Nor were attempts made to evaluate the efficiency of

local cooling on the adaptation of a man to a very uncomfortable, hot, and humid environment. The present studies were undertaken to throw light upon these problems.

Methods and Materials. The studies were conducted in an air-conditioned room with the temperature and relative humidity controlled at $75^{\circ} \pm 1^{\circ}\text{F}$ and $50\% \pm 2\%$, respectively. The temperature and relative humidity of the room could be raised within 15 minutes to the desired levels as indicated below. The room in which the subjects rested contained very little of the recording apparatus in order to reduce the psychic disturbance to a minimum. Twenty-six (25 white and one negro) young adults, trained subjects who had had consid-

erable experience around research laboratories, were used. They varied from 22 to 37 years of age. Three of the subjects were females.

The rate of water loss was measured from the skin of the right index fingertip, right forearm, and midepigastrum by a method described by Neumann, Cohn and Burch.³ The oral and rectal temperatures were measured and recorded photographically by means of copper-constantan thermocouples. The variations in the volume of pulsations of the right index fingertip and right second toetip were measured by means of a modification of

³ Neumann, C., Cohn, A. E., and Burch, G. E., *Am. J. Physiol.*, 1941, **132**, 748.

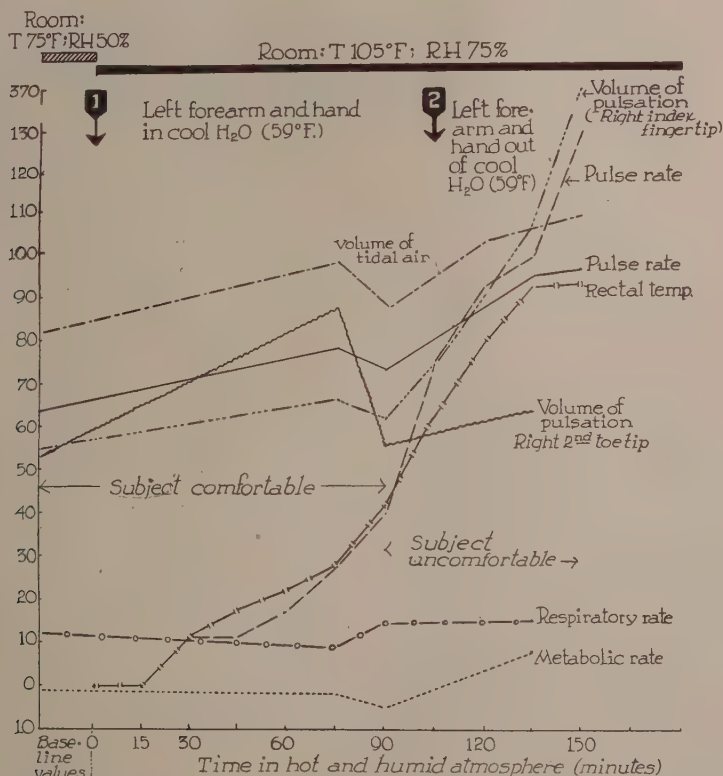


FIG. 1.

The mean effect of cooling a forearm and hand on several physiologic functions in 5 young adults resting quietly in a hot and humid room. The units of measurement indicated along the ordinate of the graph are: Water loss on sweating, mg per 5 sqcm of surface area of skin per 5 minutes; change in temperature, 0.01°C ; volume of pulsations, cumm per 5 cc of part; pulse rate, times per minute; respiratory rate, times per minute; volume of tidal air, 100 cc; metabolic rate, per cent of normal basal metabolic rate.

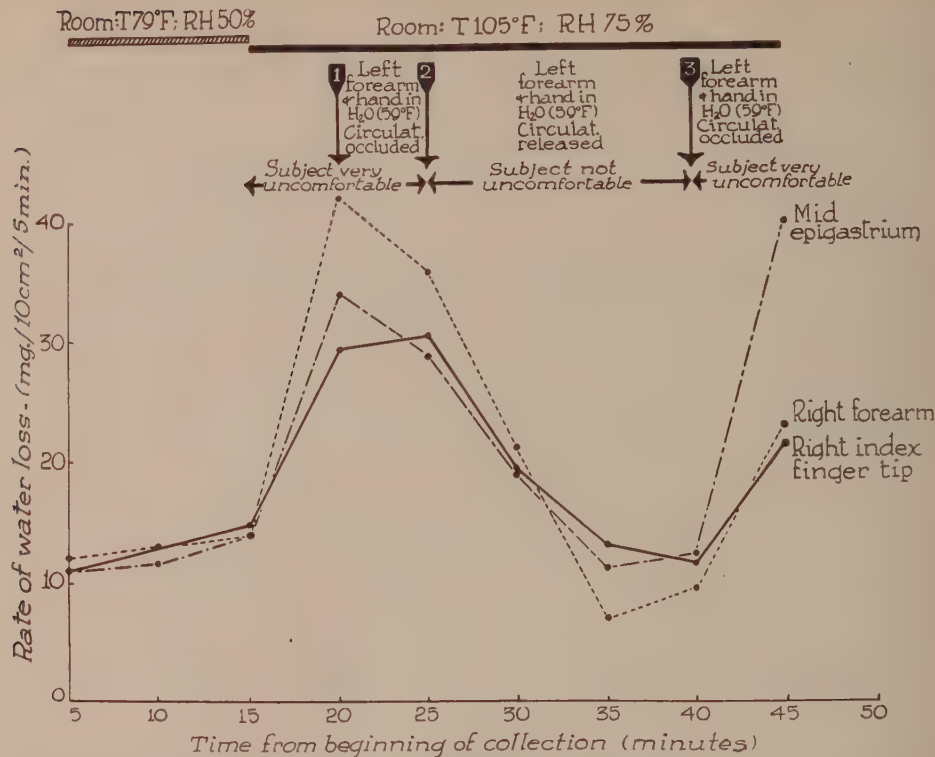


FIG. 2.

The influence of the circulation of blood in the forearm and hand cooled upon the rate of water loss from the skin of 3 areas in a representative normal young adult resting in a hot and humid environment.

the sensitive pneumoplethysmographic method of Turner.⁴ The metabolic rate and volume of tidal air were measured with the Benedict-Roth Basal Metabolism apparatus. The respiratory rate, pulse rate, and comfort were also recorded. The subject's comfort was estimated by conversation.

The subjects rested for about 30 minutes in the comfortable environment of 75°F and 50% relative humidity. The above mentioned physiologic processes were recorded simultaneously and in some instances in separate groups. The temperature and relative humidity of the room were increased within 15 minutes to 105°F and 75%, respectively. The physiologic processes were again measured with and without the left forearm and hand in a water bath maintained at 59°F. The same procedure was repeated in a dry and

hot atmosphere and with the subject in a water cooled chair. Higher room temperatures and humidities were tried. The subjects were kept in the hot and humid environment for as long as 3 hours.

Results. Some of the results are summarized by Fig. 1, 2, and 3. Absolute values are indicated in the figures.[†]

When the room temperature and relative humidity were increased from 75°F and 50% to 105°F and 75%, the subjects complained of discomfort from the heat, the pulse volume and rate increased, metabolic rate tended to drop, or not change, the volume of tidal air increased, respiratory rate decreased, sweating

[†] Ordinate units for the changes in the oral and rectal temperatures are equal to the ordinate value as indicated on the figure, times 0.01°C. In other words, the change from an ordinate reading of 0 to 90 would indicate a change of 0.90°C.

⁴ Turner, R. H., *J. Clin. Invest.*, 1937, **16**, 777.

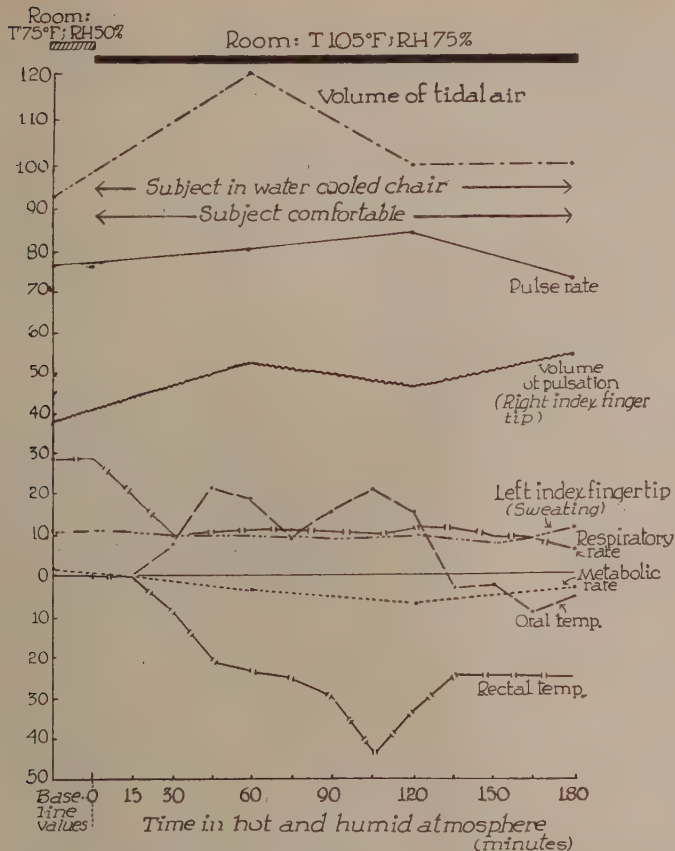


FIG. 3.

The mean effects of cooling a large portion of the body (that portion extending from the level of the inferior angles of the scapulæ above, to the junction of the middle and lower thirds of the thighs below) in a water cooled chair on certain physiologic functions in 5 young normal adults. The units of measurement shown along the ordinate are the same as for Fig. 1.

became profuse, the volume of pulsations in the finger and toetips increased, oral and rectal temperatures rose. When the left forearm and hand were placed in the cool water bath these changes were inhibited markedly, the inhibition of the changes ceasing whenever the parts were removed from the water bath (Fig. 1). The subjects were made comfortable. Occlusion of the circulation to the forearm and hand in the water bath by a blood pressure cuff removed the influence of cooling upon the physiologic processes. When the circulation was released, the parts in the cool water again inhibited the physiologic changes and the subjects again became com-

fortable. These effects upon sweating are illustrated, for a representative subject in Fig. 2.

It is seen from Fig. 1 that cooling of the left forearm and hand alone markedly inhibited, but did not completely prevent any of the physiologic changes. A larger area of body surface was cooled and isolated in a water cooled chair. Cooling and isolating that portion of the body extending from the inferior angles of the scapulæ above, to the junction of the middle and lower third of the thighs below, inhibited changes in the physiologic processes not completely inhibited by cooling a single forearm and hand (Fig. 3).

The subjects were able to remain in the hot and humid room without any discomfort for the period of 3 hours and could have remained there for longer periods without any real discomfort. The observers were unable to remain in the room for more than a few minutes at a time during the periods of observation without experiencing marked discomfort.

Comment. The data indicate that an individual resting in a hot and humid atmosphere can be made comfortable and many of his physiologic processes kept at a level for a comfortable environment by cooling a localized portion of the body. When only a small portion of the body is cooled, such as forearm and hand, the effects are definite, but not as effective as when larger areas are cooled. Cooling a localized portion of the body inhibits the accumulation of body heat. The forearm and hand represent about 5.5% of the total surface of the body,^{5,6} while the lower two-thirds of the trunk of the body and upper

two-thirds of the thighs represent about 50% of the total surface area. Obviously, since heat exchange between the environment and the body is a physical one, the larger the area of body cooled, the greater the loss of heat. However, the relatively great vascularity of the hand and fingers increases its capacity for heat transfer and thus tends to compensate for the smaller area cooled.

It is well to remember that when the parts are submerged in a cool water bath or enclosed in a cooled chair, the parts are not only sites for heat loss from the body, but also, in being isolated from the hot and humid environment, reduce the surface area exposed to the hot, humid atmosphere to absorb heat.

Since interruption of the blood supply to the cooled part with its nervous system intact failed to inhibit the production of physiologic changes by the hot and humid room, the effects of cooling the isolated part must result mainly from the maintenance of the normal body temperature and not to any significant neurogenic influences.

We wish to express our appreciation to Mr. G. Morgavi, Jr., who constructed the apparatus and participated in the studies.

⁵ DuBois, D., and DuBois, E. F., *Arch. Int. Med.*, 1915, **15**, 868.

⁶ Sawyer, N., Stone, R. H., and DuBois, E. F., *Arch. Int. Med.*, 1916, **17**, 855.

14515

Sudden Destruction of Motor End Plates by Lactic Acid.*

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The morphologic effects of lactic acid, in concentrations (0.05-0.3%) found in rigor mortis¹ of muscle, upon the labile structure of motor end plates are unknown. During post-mortem rigidity of muscle in man, monkey, and mouse there is a sudden or gradual disappearance of the end plates dependent upon the time of onset of rigor: extreme exhaustion induced by either physical activity or acute

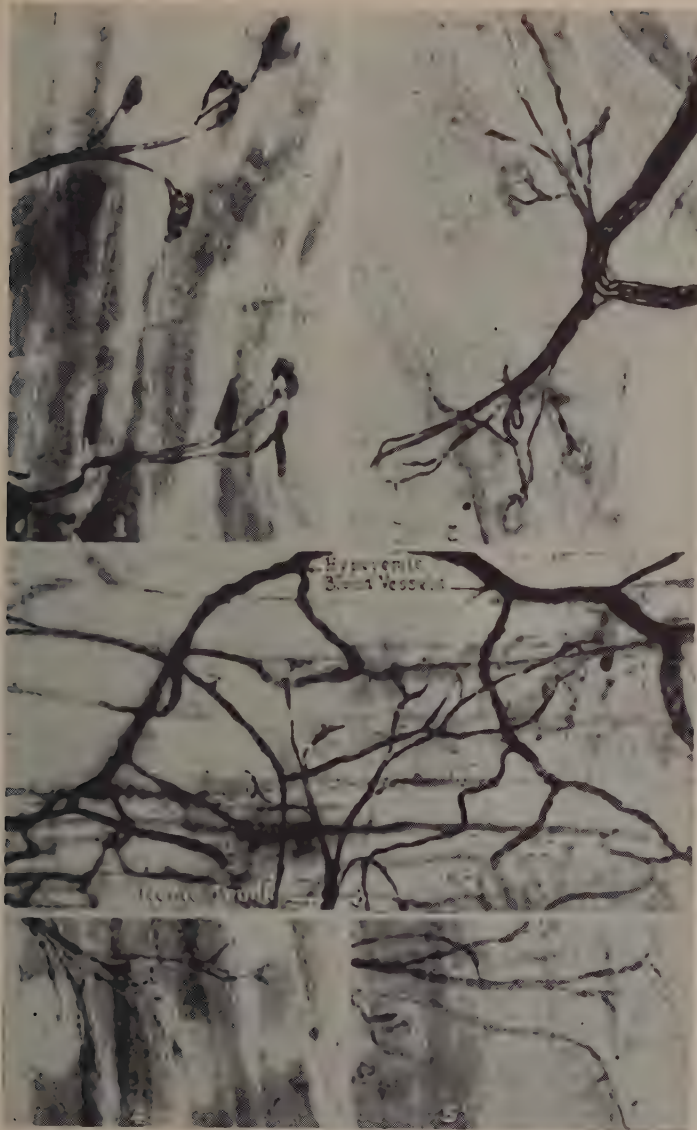
infections immediately prior to the death of the experimental animal produces both rapid onset of rigor and rapid dissolution of the end plates.² Experimental morphologic studies of the motor end plates must be made therefore before the onset of rigor and preferably from muscles excised from the living animal and processed immediately by the gold method.

Methods. Kocher collar incisions were made through the shaved skin on the ventral aspect of the neck in adult white rats, *Mus norvegicus* (250-280 g), under anesthesia by intraperitoneal injection of nembutal, 25 mg

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

¹ Fletcher, W. M., and Hopkins, F. Gowland, *J. Physiol.*, 1906-7, **35**, 247.

² Carey, E. J., unpublished observations.



FIGS. 1 TO 3

Photomicrographs, normal innervation, sternomastoid muscle, white rat, Fig. 1; motor end plates either expanded, Fig. 2, or destroyed and accompanied by hyperemic blood vessels, Fig. 3, and depletion of epilemmal axons, Figs. 4 and 5, of auripilous substances, by lactic acid. $\times 100$.

per kilo. Both sternomastoid muscles were exposed. The local zone of innervation was about 1 mm wide and more opaque than the rest of the muscle; it extended transversely mesiad to laterad and was about midway

between origin and insertion. The right muscles were removed, with bleeding controlled, run through gold technique³ and other

³ Carey, E. J., *Am. J. Path.*, 1942, **18**, 237.

histologic methods, and used as control muscles. Into the opaque zone of the exposed innervation of the left muscle, 0.1 cc of 0.05% lactic acid was injected in 10 rats; 0.1 cc of 0.10% lactic acid into the muscle of 10 rats; and 0.1 cc of 0.30% lactic acid into the muscle of 10 rats. Five rats in each series were killed within 30 seconds and the remaining 5 in each series at 25-minute intervals. The above experiments on the same number of rats were duplicated, with the same concentrations as those used in the lactic acid series, by the injection of acetic, phosphoric, betaoxybutyric, and hydrochloric acids. Although the histologic results herein reported were those obtained with lactic acid, the increase of the hydrogen ion concentration effected by the other acids produced similar morphologic results. The muscles injected with lactic acid were excised, subjected to the gold technic previously described,³ and the innervations teased out. Over 1500 gold preparations of the normal and abnormal innervations of the sternomastoid muscles in the white rat were used in this study.

Results. The normal end plates, Fig. 1, in the sternomastoid muscle of the white rat varied from 14 to 25 μ in length in the retracted state with related Kühne's granules, and from 25 to 48 μ in the expanded state with a diminution in the amount of the surrounding Kühne's granules. Immediately after the multiple injections of lactic acid into the zone of innervation the muscle grossly became opaque and shortened in a state of continuous isotonic spasm or rigor. The muscle, completely paralyzed by lactic acid, failed to respond to an electric stimulus of the spinal accessory nerve. Lactic acid, 0.05%, produced within 30 seconds retraction of many of the end plates with increased staining capacity for gold and dilatation of the epilemmal axons. This was quickly followed (30 seconds to 25 minutes) by abnormal expansion, Fig. 2, and a decrease in the quantity of Kühne's granules and in the staining capacity of the end plates with gold. The expanded hypolemmal axons were fragmented into discrete globules of various sizes progressively undergoing dissolution. The expanded end plates, continuous with dilated

and beaded epilemmal axons, were found in 1825 of 2213 end plates counted. They varied from 35 to 65 μ , measured in the longitudinal axis of the muscle fiber. About 10% of the end plates had disappeared. Lactic acid, 0.10%, destroyed 814 end plates within 30 seconds, and within 25 minutes 2946 end plates of the 3119 epilemmal axons counted were destroyed. Lactic acid, 0.3%, destroyed 972 end plates within 30 seconds, and within 25 minutes 3891 end plates of the 4000 epilemmal axons counted were destroyed. The ends of the epilemmal axons were frequently divided dichotomously and denuded, Fig. 3, of their hypolemmal end plates within 30 seconds by 0.3% of lactic acid. Close to the zones of the injected lactic acid the intramuscular blood vessels, Fig. 3, were greatly enlarged and congested with agglutinated red blood cells within 30 seconds to 25 minutes. Many muscle fibers had the characteristics of so-called Zenker's waxy degeneration. There was irregular clumping of the protoplasm and loss of the regular periodicity of the cross striations in some muscle fibers. Likewise, in many muscle fibers, there were clear oval spaces of liquefaction, Fig. 4, indicated by the terminals of the epilemmal axons denuded of their hypolemmal end plates. The sudden destruction of the end plates by lactic acid altered the local structure of the striped muscle fiber. Within 25 minutes 0.3% lactic acid not only produced dissolution of the majority of the end plates but progressive depletion or exhaustion, Figs. 4 and 5, of the gold staining substance or substances in the epilemmal axons. These exhausted epilemmal axons, Fig. 5, had ghost-like outlines. Some of the nuclei of the muscle fibers were more clearly defined than normally and were either rounded and pyknotic or enlarged by 0.3% lactic acid within 25 minutes. In local spots within the muscle there was a beginning perivascular infiltration of polymorphonuclear leucocytes within 2 hours after injection of the lactic acid.

Comments. Observations had shown that the intravascular injection of alkaline solutions in animals and of defibrinated blood in a decapitated criminal removed rigor and re-established muscle irritability;⁴ that indirect overstimulation of muscle through its

nerve supply led to Wedensky's inhibition of transmission of the nerve impulse at the motor end plates;⁵ that lactic acid caused Zenker's waxy degeneration of muscle;⁶ that the time of onset of rigor was influenced by the intact peripheral nerves;⁷ that occlusion of circulation and muscle activity produced pain and "explosive" changes in the electric potential of the hyperexcitable sensory spindles;⁸ that increased hydrogen ion concentration in muscle resulted in the discharge of augmented amounts of acetylcholine at the motor end plates;⁹ and that nerve cell proteolysis was produced by lactic acid.¹⁰

The observations in this paper suggest that the abnormal accumulation of acid metabolites of muscle activity first increase the perme-

ability of the end plates to the gold staining axonic substance, and that with increasing hydrogen ion concentration the end plates undergo complete liquefaction and act like chemical safety fuses.

Summary. Lactic acid in concentrations of 0.05 to 0.3% injected into the exposed zone of innervation of the sternomastoid muscle in the white rat produced sudden destruction of most of the motor end plates within 30 seconds to 25 minutes dependent upon the concentration of the acid. This liquefaction of the hypolemmal axons of the end plates was preceded by a first phase of retraction and a second phase of expansion and fragmentation. Lactic acid caused a progressive depletion, in both the hypolemmal and the epilemmal axons, of auriphilous substances. The blood vessels were enlarged, hyperemic, and contained clumps of agglutinated red blood cells in the rigorous muscle paralyzed by lactic acid. It is suggested that the acid metabolites which accumulate during rigor mortis of muscle are responsible for the destruction of the end plates and that during life certain processes that result in the intramuscular accumulation of acid, such as vascular congestion and anoxia, likewise may result in the destruction of the motor end plates.

⁴ Brown-Séquard, E., *Compt. Rend. et Mémoires de la Soc. Biol.*, 1851, **3**, 147; *Arch. de Physiol.*, 1889, **21**, 675; *ibid.*, 1889, **21**, 726.

⁵ Wedensky, N. E., *Arch. f. d. ges. Physiol.*, 1903, **100**, 1.

⁶ Wells, H. Gideon, *J. Exp. Med.*, 1909, **11**, 1.

⁷ Norris, Richard, *J. Anat. and Physiol.*, 1867, **1**, 217.

⁸ Matthews, B. H. C., *J. Physiol.*, 1933, **78**, 1.

⁹ Gesell, R., Brassfield, C. R., Hansen, E. T., and Mason, A., *Science Supplement*, 1942, **95**, 11.

¹⁰ Rosenbohm, A., *Biochem. Z.*, 1936, **289**, 279.

14516

Excretion of Metabolic Products of Sulfapyridine in the Dog.

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It was suggested that, following the oral administration of sulfapyridine in man, water-soluble detoxication products of the drug appeared in the urine.¹ Later it was reported that administration of the drug caused a marked increase in the urinary glucuronides, and the glucuronic acid output paralleled the sulfapyridine levels.² The isolation of a

hydroxysulfapyridine and a water-soluble hydroxysulfapyridine glucuronide was announced.³ Details of this work are presented here.

Experimental. Daily doses of 0.5 g per kg body weight of sulfapyridine were fed to dogs and urine samples, collected from time to time, were worked up as follows:

Isolation of the hydroxysulfapyridine: 2400 cc of urine containing 18.0 g of sulfa-

¹ Ratish, H. D., Bullowa, J. G. M., Ames, J. B., and Seudi, J. V., *J. Biol. Chem.*, 1939, **128**, 279.

² Seudi, J. V., Ratish, H. D., and Bullowa, J. G. M., *Science*, 1939, **89**, 516.

³ Seudi, J. V., *Science*, 1940, **91**, 486.

pyridine as measured by the method of Bratton and Marshall⁴ was acidified with acetic acid and cleared with lead acetate. The filtrate was neutralized with dilute ammonia and treated with a saturated solution of basic lead acetate to maximum precipitation. The filtrate, containing 4.5 g of sulfapyridine, was discarded. The basic lead was removed as the sulfide. The filtrate was saturated again with hydrogen sulfide to complete the removal of lead, and the filtrate (pH 5.0-5.5) was concentrated *in vacuo* at 30° to 35° to a thick syrup. Extraction with chloroform gave 125 mg of crystals. Recrystallized several times from water and dilute alcohol, the hydroxysulfapyridine appeared as pale yellow needles and melted at 180-181° corr. It depressed the melting point of sulfapyridine; gave a positive diazo, ferric chloride, and indophenol reaction.⁵ The ultra-violet absorption data obtained with aqueous solutions of the hydroxysulfapyridine have been published.²

Calculated for $C_{11}H_{11}O_3N_3S$:

C = 49.81; H = 4.15; N = 15.84

Found:

C = 50.33; H = 4.26; N = 15.96

Isolation of the Silver Salt of the Hydroxysulfapyridine Glucuronide: 250 cc of urine containing 5.0 g of sulfapyridine as measured by the Marshall method⁴ was treated with powdered lead acetate to maximum precipitation and the precipitate was discarded. The filtrate, neutralized with dilute ammonia, was treated with saturated basic lead acetate to maximum precipitation. The precipitate was slurried in water and the lead was removed as the sulfide. The filtrate was freed of excess hydrogen sulfide by aeration and the product was precipitated with an excess of mercuric acetate. The mercury precipitate was mixed to a paste with small volumes of water and the mercury was removed with hydrogen sulfide. After removing excess hydrogen sulfide, the product was precipitated by the addition of excess 10% silver nitrate. The silver salt of the hydroxysulfapyridine glucuronide was immediately removed by cen-

trifugation and was thoroughly washed with water, alcohol and ether. Dried at 100° it weighed 154 mg. The product, a pale yellow powder, gave a diazo⁴ and a naphthoresorcinol value⁶ corresponding to approximately one molecule of glucuronic acid per molecule of sulfapyridine. The indophenol reaction was negative but became positive after acid hydrolysis.

Calculated for

$C_{11}H_9O_2N_3S \text{ Ag} \cdot O \cdot C_6H_8O_6Ag \cdot 3H_2O$:

C = 28.77; H = 3.24; N = 5.92;

Ag = 30.46; H_2O = 7.61

Found:

C = 28.98; H = 3.38; N = 5.73;

Ag = 30.16; H_2O = 6.85

Isolation of the Brucine Salt of the Hydroxysulfapyridine Glucuronide: On several occasions it was not possible to isolate analytically pure samples of the silver salt. On one occasion the above procedure gave a silver salt containing 32% silver (1.75 g of the silver salt were obtained from 1350 cc of urine containing 13 g of arylamine). This was powdered and suspended in 10 cc of water and the silver was removed as the sulfide. The filtrate was concentrated to a syrup *in vacuo*, and the syrup was taken up in 95% alcohol. Addition of an excess of brucine in alcohol yielded the brucine salt of the hydroxysulfapyridine glucuronide. The product, pale yellow needles, melted with decomposition at 215°

Calculated for

$C_{17}H_{18}O_9N_3S \cdot C_{28}H_{26}N_2O_4 \cdot 4H_2O$:

C = 52.98; H = 5.74; N = 7.72

Found:

C = 53.03; H = 6.03; N = 7.84

The position of the hydroxyl group in the hydroxysulfapyridine has not been completely established. The indophenol reaction⁶ is given only by those phenols possessing a free para

⁶ Maughan, G. B., Evelyn, K. A., and Browne, J. S. L., *J. Biol. Chem.*, 1938, **126**, 567.

* The sulfonamide hydrogen forms a silver salt. Adding one molecule of silver nitrate to a hot ammoniacal solution of sulfapyridine gives excellent yields of the white crystalline silver salt. Calculated for $C_{11}H_{10}O_2N_3S \text{ Ag}$: Ag = 30.33. Found: Ag = 30.07.

⁴ Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

⁵ Seudi, J. V., *J. Biol. Chem.*, 1941, **139**, 707.

position, and it is not given by pyridones. Hence the hydroxyl group can only be in the 3-position of the pyridine ring or in the benzene ring ortho or meta to the primary amine. Through the courtesy of Dr. A. R. Day, who prepared the ortho hydroxy derivative of sulfapyridine, we have been able to rule out this possibility. The meta hydroxy derivative has not been ruled out, but we have been inclined to favor the 3-position in the pyridine ring as being the more plausible. This postulation is in agreement with the recent work of Weber *et al.*⁷ which definitely places the hydroxyl group in the pyridine ring.

Discussion. These results indicate that the

urinary elimination of sulfapyridine is more complex than hitherto recognized. Since the hydroxysulfapyridine and the water-soluble glucuronide possess free arylamino groups they are measured by diazotization procedures along with the "free" sulfapyridine. Their therapeutic efficiency may not be equivalent to that of the unchanged drug. Further, the excretion of a part of the drug in a highly soluble form is important in the etiology of sulfapyridine urolithiasis.⁸

Summary. Following oral administration of sulfapyridine, a hydroxysulfapyridine and a water-soluble hydroxysulfapyridine glucuronide have been isolated from dog urine.

⁷ Weber, C. J., Lalich, J. J., and Major, R. H., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 190.

⁸ Seudi, J. V., and Robinson, H. J., *Am. J. Med. Sci.*, 1941, **201**, 711.

14517

Microbiological Differentiation of Pyridoxine and Pseudopyridoxine.*

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Snell, Guirard, and Williams¹ obtained abnormally high pyridoxine values when urine was assayed with *Streptococcus lactis R* against a pyridoxine standard. These workers demonstrated that the effect was due to the presence in urine of a metabolite of pyridoxine which they called pseudopyridoxine. It was found that pseudopyridoxine could be formed by the interaction of pyridoxine and amino acids during autoclaving.²

Using yeasts and molds for pyridoxine assay, other workers (Robbins and Ma,³ Carpenter

et al.,⁴ Atkin *et al.*,⁵ and Stokes *et al.*,⁶) did not obtain abnormally high values on materials known to contain pseudopyridoxine. It has therefore been postulated that these organisms do not respond to pseudopyridoxine.

While Snell *et al.*² obtained evidence that *S. lactis* utilizes only pseudopyridoxine and not pyridoxine, no conclusive evidence has been obtained that yeasts do not utilize both pyridoxine and pseudopyridoxine. The high assay values obtained with *S. lactis* merely reflect the small conversion of pyridoxine to pseudopyridoxine in the standard. If both compounds are active for an organism, abnormally high assay values would not be expected.

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Illinois.

¹ Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, 1942, **143**, 519.

² Snell, E. E., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 356; *J. Bact.*, 1943, **45**, 199.

³ Robbins, W. J., and Ma, R., *Proc. Nat. Acad. Sci., U. S.*, 1942, **29**, 172.

⁴ Carpenter, L. E., Elvehjem, C. A., and Strong, F. M., *Proc. Soc. Exp. Biol. and Med.*, 1943, **54**, 123.

⁵ Atkin, L., Schultz, A. S., Williams, W. L., and Frey, C. N., *Ind. Eng. Chem., Anal. Ed.*, 1943, **15**, 141.

⁶ Stokes, J. L., Larsen, A., Woodward, C. R., Jr., and Foster, J. W., *J. Biol. Chem.*, 1943, **150**, 17.

In order to obtain specific evidence as to whether yeasts can utilize pseudopyridoxine, we have made the two studies reported below.

Experimental. The following work was carried out on human sweat which was found to contain pseudopyridoxine as well as pyridoxine.

The method used for the separation of pyridoxine and pseudopyridoxine in sweat involved the elimination of pyridoxine by yeast growth, filtration of yeast cells, and assay of the filtrate for pseudopyridoxine. In a similar manner pseudopyridoxine could be eliminated by bacterial growth, the bacterial cells removed by filtration, and the filtrate assayed for pyridoxine.

A sample of pooled filtered sweat, concentrated 2 to 1, contained 0.0018 μg of pyridoxine per cc by the *Saccharomyces carlsbergensis* procedure of Atkin, Schultz, Williams, and Frey,⁵ and 4 μg of pyridoxine equivalents of pseudopyridoxine using the *Lactobacillus casei* procedure of Carpenter, Elvehjem, and Strong.⁴

Four cc of this filtered sweat and 1 cc of *Sacch. carlsbergensis* inoculum were added to 5 cc of the medium of Atkin *et al.*⁵ Less than maximum growth of the yeast was obtained in 24 hours. The yeast cells were filtered off and the filtrate assayed with *L. casei*. Four μg per cc of sweat of pyridoxine equivalent of pseudopyridoxine were found in the filtrate. The same medium after filtering off the yeast cells was reinoculated with yeast and no growth was obtained, indicating that the pyridoxine had been completely utilized by the first yeast growth. When 5 and 20 $\text{m}\mu\text{g}$ of pyridoxine were added to the filtrate 99% was recovered, showing that growth had not been prevented by the presence of any inhibitory substances which might have been formed by the original yeast growth.

L. casei was grown on the medium of Landy and Dicken,⁷ omitting the pyridoxine, and the same sweat as before was added at 10 levels from 0.1 to 5 cc per tube. After 72 hours the cells were removed by filtration. The pyridoxine was recovered from the filtrate by adsorption and elution from Lloyd's reagent and

the amount found by yeast assay was 0.0016 μg per cc of sweat. This value corresponds closely to the value of 0.0018 μg per cc obtained on the original sweat sample. Another aliquot of the casei filtrate was re-inoculated with *L. casei* and no growth was obtained. When pseudopyridoxine[†] was added, growth of *L. casei* was obtained.

These experiments demonstrate that yeast can use pyridoxine and not pseudopyridoxine, whereas *L. casei* uses only pseudopyridoxine and not pyridoxine.

The assay values on sweat obtained by the yeast *Sacch. carlsbergensis* correspond with those obtained by *Sacch. oviformis*⁸ and the mutant mold *Neurospora sitophila* 299.⁹ Apparently therefore, these organisms also respond only to pyridoxine. In addition to *L. casei*⁴ and *S. lactis* R,^{1,2} *Leuconostoc mesenteroides* was also found to respond to pseudopyridoxine and therefore cannot be used as an assay organism for pyridoxine as suggested by Gaines.⁹

Since *L. casei* uses only pseudopyridoxine, it could be used for the assay of this compound if pseudopyridoxine were available for a standard. An assay using pyridoxine, itself, as a standard is not accurate due to the variability of the conversion of pyridoxine to pseudopyridoxine during sterilization. This fact was shown by assaying sweat samples for pseudopyridoxine with *L. casei* using three different sterilization times, 10, 15, and 20 minutes. The assay values obtained were respectively, 10, 4, and 0.13 μg per cc of pyridoxine equivalent of pseudopyridoxine. The assay values decreased as more of the pyridoxine in the standard was converted to pseudopyridoxine.

Work was carried out on the separation of pyridoxine and pseudopyridoxine without recourse to microorganisms and some success was obtained with Lloyd's reagent.

Fifty cc of a pooled sample of sweat were

† By adding pyridoxine in large amounts, 0.1 to 1.0 μg per tube before sterilization. Amounts of pyridoxine less than 0.1 γ /tube do not give any stimulation of *L. casei* at the sterilization times used here (up to 20 minutes).

⁸ Burkholder, P. R., *Am. J. Bot.*, 1943, **30**, 206.

⁹ Gaines, S., and Stahly, G. L., *J. Bact.*, 1943, **46**, 441.

⁷ Landy, M., and Dicken, D. M., *J. Lab. Clin. Med.*, 1942, **27**, 1086.

TABLE I.
Separation of Pyridoxine and Pseudopyridoxine by
Means of Lloyd's Reagent.

	Filtrate, γ/cc	Eluate, γ/cc
Pyridoxine		
<i>Sacch. carlsbergensis</i>	0.0000	0.0016
Pseudopyridoxine		
<i>L. casei</i>	0.16 equivalents	0.31 equivalents

brought to pH 3.0 with sulfuric acid. 0.5 g of Lloyd's reagent was added to absorb the pyridoxine. The pyridoxine was eluted with 15 cc (three 5-cc portions) of 1/10 N NaOH. This eluate was neutralized and diluted to 25 cc. Both the solution left after the Lloyd's reagent absorption (concentrated to 25 cc) and the eluate were analyzed for pyridoxine

and for pseudopyridoxine. The results are given in Table I.

From this table it can be seen that while about two-thirds of the pseudopyridoxine follows the pyridoxine in its absorption as found by Snell *et al.*,¹ the filtrate does contain considerable pseudopyridoxine and no pyridoxine. Thus some separation was secured and again the yeast was unable to utilize the pseudopyridoxine.

Summary. Pyridoxine has been separated from pseudopyridoxine by using the yeast *Saccharomyces carlsbergensis* and the bacterium *Lactobacillus casei*. It has been shown that *Sacch. carlsbergensis* assays and utilizes only pyridoxine while *L. casei* assays and utilizes only pseudopyridoxine.

14518 P

Ovarian Tumors in Adult Rats Following Prepuberal Administration of Estrogens.*

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Estrogens injected into lactating rats have been shown to be transmitted to the suckling young inducing changes in urogenital organs of both sexes.¹ The effects are of such a nature that it seemed advisable to make further observations on similar animals, at intervals after cessation of treatment, in order to determine the permanency of the condition and the course of any subsequent changes. It has been reported that female rats given prepuberal injections of androgen^{2-4,6} or estro-

gen⁵⁻⁷ have severe reproductive deficiencies as adults. Because of variations in methods of administration, hormones used, and length of treatment, the present study brings out differences thought to be of significance.

Methods. We have observed that in untreated female littermates of immature animals given daily injections of diethylstilbestrol, the vaginas open at the same time as in injected animals. This has been confirmed repeatedly with large litters containing only one injected animal. Similar changes have been observed in litters confined to cages in which stilbestrol was sprayed upon shavings used as nesting material. Injection of cagemates or contamination of nesting material with stilbestrol may therefore be used as alternative methods of administering hormone to immature animals.

* The authors are indebted to Dr. O. Kamm of Parke, Davis and Co. for generous supplies of theelin and to Dr. G. A. Harrop of the Squibb Institute for Medical Research who kindly furnished the stilbestrol used in this investigation.

¹Weichert, C. K., and Kerrigan, S., *Endocrinology*, 1942, **30**, 741.

²Wilson, J. G., Young, W. C., and Hamilton, J. B., *Yale J. Biol. and Med.*, 1940, **13**, 189.

³Wilson, J. G., Hamilton, J. B., and Young, W. C., *Endocrinology*, 1941, **29**, 784.

⁴Greene, R. R., Burrill, M. W., and Ivy, A. C., *Anat. Rec.*, 1942, **83**, 19.

⁵Wilson, J. G., *Anat. Rec.*, 1943, **86**, 341.

⁶Greene, R. R., and Burrill, M. W., *Proc. Am. Physiol. Soc.*, 1941, 114.

⁷Turner, C. D., *Proc. Am. Physiol. Soc.*, 1941, 283.

Five groups of rats, totalling 154 animals, were treated with estrogen for various lengths of time, the initial treatment being given at various times between birth and the 28th day of life. Animals of Group I were suckled by mothers injected with .1 mg stilbestrol daily; in those of Group II the mothers received .5 mg estrone daily. Group III received direct injections of .01 mg stilbestrol, while those of Group IV were affected by stilbestrol injected into littermates given .01 mg daily. Group V consisted of animals exposed to small quantities of environmental stilbestrol on nesting material. Periods of treatment for the first 4 groups ranged from 1 to 14 days, for Group V, 21 or more days. Vaginal smears were taken routinely. Representatives from each group were autopsied either at the end of treatment, at intervals following treatment, or at mature ages ranging from 4 to 15 months.

Results. Abnormalities appeared in the smear histories in all groups. The degree of abnormality was related to the age at which treatment was begun since it was less pronounced and appeared only after 4-9 months among animals treated at the later ages. Characteristically, these animals tended to remain in vaginal estrus for extended periods. In many, a peculiar type of smear, which often persisted for months, contained both large epithelial cells and leucocytes in quantity. Since this was maintained over extended periods it cannot be regarded as a transitional stage but would seem to be the result of continued estrogenic stimulation associated with some other unknown condition.

The majority of these prepuberally treated animals failed to breed. In many, however, copulations occurred repeatedly. Spermatozoa were often noted in the vaginal smears, and sperm plugs were also observed. The few animals which did produce young, in almost every case, came from litters in which treatment was begun late. Some of these subse-

quently failed to breed and either developed irregular cycles or permanent vaginal estrus. Some animals exposed to stilbestrol sprayed repeatedly on shavings between days 8 and 20 maintained regular cycles for over 400 days while caged with males but, nevertheless, failed to become pregnant.

Few gross changes were observed in reproductive organs of animals under 120 days of age, but after this time marked changes began to appear in ovaries, uteri, and Fallopian tubes. The most pronounced alteration was the formation of roughly spherical or multilobular cysts in one or both ovaries, which in some cases were larger than the stomach. These cysts contained a great amount of yellowish caseous material of a semi-fluid consistency. Gross sections, made after fixation, showed a firm outer wall enclosing a relatively large granular mass. In animals less than 6 months old the enlargement was generally of a slighter degree. In a few cases ovaries were atrophic. Some of these were taken from animals after 40 to 60 days of persistent vaginal estrus. A considerable amount of tissue often connected the enlarged ovaries to the viscera and to the body wall, which in 2 cases became necrotic. Histological and bacteriological studies are being carried on and will be reported later. The following abnormal conditions have been identified: pyo-ovarium, pyometra, pyosalpinx, and uterine metaplasia. No typical ovarian tissue has been seen in the greatly enlarged ovaries studied thus far.

Our results differ from those of other investigators⁵⁻⁷ who have failed to report a cystic ovarian condition in rats following similar treatment. In size and gross characteristics, these ovaries resemble those of animals given large amounts of testosterone propionate or estrone over prolonged periods.⁸

⁸ Shay, H., Gershon-Cohen, J., Paschkis, K. E., and Fels, S. S., *Endocrinology*, 1939, **25**, 933.

Viability of a Transmissible Fowl Tumor (Olson) upon Storage at Low Temperatures.

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A fowl tumor under study at this Laboratory during the past year and one-half was obtained through the courtesy of Dr. Carl Olson, Jr. It was described¹ as a transmissible lymphoid tumor having cells with "relatively large vesicular nuclei and distinct nucleoli." That the tumor appears to be highly virulent is indicated by the high tumor incident when transplants to the pectoral muscle are made and by the rapid rate at which it can be serially transferred.^{1,2} Its malignancy is indicated by the occurrence of early and extensive metastases.^{1,2} Transmission by a cell-free inoculum has thus far not been demonstrated.

It is well known^{3,4} that the agents producing neoplasms in the fowl will survive low temperatures for a considerable period, and that most^{5,6,7} but not all mammalian neoplasms will survive freezing under certain conditions. The viability of this tumor¹ after storage has thus far not been reported. If inocula can be frozen and stored under certain conditions, the work of maintaining a constant and known supply for experimental use can be materially reduced. To examine this possibility we have tested several samples of inoculum from different transfers for the ability of the minced tumor to survive low temperatures.

Procedure. Tumors grown in the pectoral muscle of chickens maintained in serial transfer (7-day interval) were excised aseptically,

minced with scissors or a tissue mincer and placed in pyrex glass tubes and Lusteroid* tubes. The former were sealed by means of a gas-oxygen flame, the latter with screw cap and adhesive tape. The samples were frozen by immersing the tubes in 95% ethyl alcohol and lowering the temperature between 0°C and -20°C at the rate of 1° per minute by the addition of small pieces of solid CO₂. The temperature was then reduced more rapidly to -60°C by the addition of larger pieces of solid CO₂. This method is similar to that described by Breedis⁸ for the slow freezing of leukemic cells. After freezing, the tubes were transferred to a CO₂ icebox having a temperature range of -65°C at the top and -76°C at the bottom of the box. Tumor samples were thawed just prior to inoculation by placing the tubes under running tap water.

Tumor samples were tested by diluting minced⁹ tumor with equal parts of Tyrode's solution, saline, or heavy mineral oil, after which 1 cc of the suspension was injected into the pectoral muscle.

Results and Discussion. Results of viability tests on tumors from 9 different donors are given in Table I. All of the tumor samples tested before freezing produced takes in all birds inoculated with the exception of Nos. IV and VI (25% and 80% takes respectively). These are typical of results of other inoculations of this tumor (unfrozen) at the time these samples were obtained. The tumor samples tested after freezing by the method outlined and inoculated immediately (without storage) gave no indication of a decrease in activity.

¹ Olson, Carl, Jr., *Cancer Res.*, 1941, **1**, 384.

² Unpublished data obtained at this Laboratory.

³ Rous, P., and Murphy, J. B., *J. Exp. Med.*, 1914, **19**, 52.

⁴ Furth, J., *J. Exp. Med.*, 1932, **55**, 495.

⁵ Snell, G. D., and Cloudman, A. M., *Cancer Res.*, 1943, **3**, 396.

⁶ Breedis, C., and Furth, J., *Science*, **88**, 531.

⁷ Mider, C. B., and Morton, J. J., *Am. J. Cancer*, 1939, **35**, 502.

* Obtained from Lusteroid Container Co., South Orange, N.J.

⁸ Breedis, C., *J. Exp. Med.*, 1942, **76**, 221.

⁹ Olson, Carl, Jr., *Am. J. Vet. Res.*, 1941, **2**, 295.

TABLE I.
Viability of Minced Tumor after Freezing and Storage at -65° to -76°C .

Donor	Transfer No.	Tumor incidence T/I*			Storage period (days)
		Fresh inoculum not frozen	Inoculum frozen only	Inoculum frozen and stored	
I	1	12/12	—	2/6	28
II	3	—	—	3/3	10
III	4	12/12	—	7/7	182
				7/7	391
IV	4	1/4	2/4	8/8	384
V	6	3/3	—	2/7	369
VI	10	4/5	—	7/7	328
VII	12	4/4	3/3	8/8	102
				2/7	307
VIII	18	3/3	—	7/8	245
IX	20	4/4	—	8/8	225

*T—No. of birds that developed a tumor at site of inoculation.

I—No. of birds inoculated.

Samples tested after freezing and storing for 10 to 391 days in a CO_2 icebox produced 100% takes in 7 of 11 tests. Two inoculations showed an apparent increase in activity; whereas, 4 tests showed a decrease after storage. One of these, sample VII, produced tumors in all of the 8 birds inoculated after 102 days of storage but only 2 of 7 birds developed tumors after 307 days of storage. However, there appeared to be no definite relation between the length of period stored and loss in activity. The minced tumors that showed a decrease in activity were among those stored in Lusteroid tubes, while those stored in pyrex glass produced takes in all birds inoculated with tumor stored up to 391 days. It may be possible that inadequate seals were obtained with screw cap and tape and thus sufficient CO_2 was allowed to enter some of the Lusteroid tubes and partially inactivate

the minced tumor.

Tumors obtained from recipients of 4 of the storage tests described were transferred in one case in the fresh unfrozen state, and in 3 other cases after the tumors had been frozen and stored for 22 to 229 days.

Typical tumors developed in all of 34 birds inoculated. Thus, the storage of minced tumor tissue at low temperatures has no apparent effect upon its ability to produce typical tumors after one passage in birds or upon its viability during low temperature storage after one transfer in birds.

Conclusion. When the transmissible fowl tumor (Olson) was frozen slowly and stored at -65°C to -76°C for 10 to 391 days, no significant reduction or alteration in its capacity to produce typical tumors in the pectoral muscle was detected by methods employed.

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"Folic Acid" a Tumor Growth Inhibitor.

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Studies on tumor growth inhibitors, carried out in this laboratory, suggested the possible importance of "folic acid" for malignant growth. Evidence has recently been presented that inositol, another member of the

vitamin B-complex, inhibited tumor growth, whereas 8 other crystalline B-vitamins were ineffective.¹

In the following we report experiments in which the action of a "folic acid concentrate"*

and the action of the crystalline *L. casei* factor^{2†} on tumor growth was investigated. In these studies a rapid test for tumor growth inhibitors was employed, as previously described.³ In this test the inhibitory action is judged by comparing tumor sizes and tumor weights of matched treated and untreated groups of mice within an experimental period of 48 hours.

In Table I one experiment is presented, in which varying doses of the "folic acid concentrate" were studied. In Table II one experiment is presented, in which varying doses of *L. casei* factor were studied.

It is evident from these tables that "folic acid concentrate" and *L. casei* factor are

TABLE I.

Effect on Tumor Growth of 4 Intravenous Injections of "Folic Acid Concentrate" in Varying Doses Given Over a Period of 48 Hours.* Seven animals were used in each group.

Group No.	Dose of "folic acid"† γ	Mean terminal tumor wt., mg	Standard error, mg
805	0	951	88
803	0.16	616	16
802	0.32	566	57
801	0.63	509	47
800	1.25	470	45
799	2.50	386	42
798	5.00	333	67

* Female Rockland mice transplanted with Sarcoma 180; start of the experiment 8 days after transplantation; mice kept on normal diet.

† Micrograms of material of "potency" 40000 (Williams standard, as measured by *L. casei* assay.)

¹ Laszlo, D., and Leuchtenberger, C., *Science*, 1943, **97**, 515.

* We received the "folic acid concentrate" through the courtesy of Dr. J. C. Keresztesy of the Research Laboratories of Merck and Co., Inc.

² Stokstad, E. L. R., *J. Biol. Chem.*, 1943, **149**, 573.

† The *L. casei* factor was obtained through the courtesy of Dr. B. L. Hutchings and Dr. E. L. R. Stokstad of Lederle Laboratories, Inc.

³ Laszlo, D., and Leuchtenberger, C., *Cancer Research*, 1943, **3**, 401.

TABLE II.

Effect on Tumor Growth of 4 Intravenous Injections of *L. casei* Factor in Varying Doses Given Over a Period of 48 Hours. Seven animals were used in each group.

Group No.	Dose of <i>L. casei</i> factor γ	Mean terminal tumor wt., mg	Standard error, mg
828	0	893	78
827	.0063	704	37
826	.0125	650	60
825	.0250	540	23
824	.0500	517	36
823	.1000	469	48
822	.2000	394	21
821	.4000	333	18

* Female Rockland mice transplanted with Sarcoma 180; start of the experiment 8 days after transplantation; mice kept on normal diet.

strong inhibitors of tumor growth. In these experiments 0.16 γ "folic acid concentrate" and 0.025 γ *L. casei* factor inhibited tumor growth significantly in a group of 7 mice. Similar results were obtained with "folic acid concentrate" in 6 experiments on 117 mice and with *L. casei* factor in 10 experiments on 364 mice. On weight basis, the *L. casei* factor is a many hundredfold stronger inhibitor than inositol.

Pollack, Taylor, and Williams⁴ reported that the vitamin present at the highest relative level in both human and rat cancer (the same seems to be true for the mouse) is folic acid. According to their Table 14 the relative inositol level is next to that of folic acid, while pyridoxine and biotin are present at relatively low levels. It seems of interest to investigate the possible relationship between the inhibitory action of folic acid and inositol and their relatively high content in malignant tumors.

Summary. A "folic acid concentrate" and a crystalline *L. casei* factor were found to be strong inhibitors of tumor growth.

⁴ Pollack, M. A., Taylor, A., and Williams, B. J., *Studies on B Vitamins in Human, Rat, and Mouse Neoplasms*, The University of Texas Publication, 1942, 4237, 56.

Failure to Demonstrate Alkaline Phosphatase Activity in Inclusion Bodies by the Histochemical Technic.*

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Phosphatase activity of purified elementary bodies of vaccinia was reported by Macfarland and Salaman¹ and confirmed by Hoagland, Ward, Smadel, and Rivers.² The first authors concluded from their experiments that the enzymatic activity was due to the virus and not to tissue contaminants, but Hoagland *et al.* found that vaccinia virus may adsorb large quantities of phosphatase from a dilute solution containing the crude enzyme, and considered this strong evidence for the adsorption hypothesis. In conclusion they said, "Until some method can be devised which will distinguish between the enzymes of the host cell and those which may be an integral part of the virus it would seem that the problem of the enzyme constitution of vaccinia virus is incapable of definite solution."

With a full realization of the physical and constitutional differences between the elementary body of vaccinia virus and its inclusion body, histochemical preparations of phosphatase activity of the vaccinia, herpes simplex, fowlpox, and tracheo-laryngitis inclusion bodies were studied.

Gomori³ and Takamatsu⁴ independently introduced an ingenious histochemical technic for the demonstration of phosphatase activity on ordinary histologic slide preparations. Taking advantage of the fact that this enzyme was preserved in alcohol fixation and paraffin sections, they showed that incubation in a solution containing sodium- β -glycero-

phosphate and calcium chloride buffered at a suitable pH caused the precipitation of calcium phosphate at the site of enzymatic activity. It was then possible to stain for the insoluble calcium phosphate. This technic has the advantage of preserving tissue integrity, revealing clearly the phosphatase activity of the host tissue. It appears that Gomori's technic, although not quantitative, is just as specific for alkaline phosphatase as are the usual biochemical methods of determination.^{5,6,7} Gomori has introduced a comparable method for the visualization of acid phosphatase⁸ and small amounts of the enzyme can be demonstrated by both the acid⁹ and alkaline¹⁰ technic.

It seemed reasonable, therefore, that if vaccinia inclusion bodies failed to give a positive histochemical phosphatase reaction when studied *in situ*, the tissue contamination hypothesis might be given added weight. If, however, inclusion bodies stained positively no interpretation could be made since the adsorption phenomenon might occur directly in the tissues. By the use of the same technic Willmer¹¹ has shown that the chromosomes of dividing cells in tissue cultures give a strongly positive reaction for the presence of phosphatase. It has been shown that alkaline phosphatase prepared from intestinal mucous membrane is capable of hydrolyzing yeast and virus nucleic acid but has no effect on tobacco mosaic virus.¹²

* Submitted in partial fulfillment of the degree of Doctor of Philosophy in the Graduate School of the University of Minnesota.

¹ Macfarlane, M. G., and Salaman, M. H., *Brit. J. Exp. Path.*, 1938, **19**, 184.

² Hoagland, C. L., Ward, S. M., Smadel, J. E., and Rivers, T. M., *J. Exp. Med.*, 1942, **76**, 163.

³ Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 23.

⁴ Takamatsu, H., *Tr. Japanese Path. Soc.*, 1939, **29**, 492.

⁵ Gomori, G., *J. Cell. and Comp. Physiol.*, 1941, **17**, 71.

⁶ Gomori, G., *Am. J. Path.*, 1943, **19**, 197.

⁷ Landow, H., Kabat, E., and Newman, W., *Arch. Neurol. and Psychol.*, 1942, **48**, 518.

⁸ Gomori, G., *Arch. Path.*, 1941, **32**, 189.

⁹ Wilmer, H. A., *J. Exp. Med.*, 1943, **78**, 225.

¹⁰ Wolf, A., Kabat, E. A., and Newman, W., *Am. J. Path.*, 1943, **19**, 423.

¹¹ Willmer, E. N., *J. Exp. Biol.*, 1942, **19**, 11.

¹² Cohen, S. S., and Stanley, W. M., *J. Biol. Chem.*, 1942, **142**, 863.

TABLE I.
Failure to Demonstrate Alkaline Phosphatase in
Inclusion Bodies.

Virus	Source inclusion	Histochemical reaction
Vaccinia	rabbit cornea	negative
"	" "	"
"	chorio-allantoic membrane	"
"	chorio-allantoic membrane	"
Fowlpox	chicken epidermis	"
Herpes simplex	chorio-allantoic membrane	"
Tracheo-laryngitis	chorio-allantoic membrane	"

The corneas of 2 rabbits were inoculated with vaccinia virus and the rabbits killed after 24 and 48 hours respectively. Vaccinia virus was inoculated into the chorio-allantoic membrane of 2 chick embryos of 12 days and allowed to incubate for 6 and 7 days. Similar preparations were made with herpes simplex

and tracheo-laryngitis virus. A section of epidermis from a chicken dead of naturally occurring fowlpox was studied. Each of these histochemical preparations revealed the presence of their corresponding large inclusion bodies, but in none of the inclusions was there any apparent alkaline phosphatase activity. Likewise, there was little or no phosphatase activity in the chorio-allantoic membrane in the immediate vicinity of the virus. The lesions in skin of the chicken, however, showed slight and scattered enzyme activity.

Summary. We failed to demonstrate alkaline phosphatase in the inclusion bodies of vaccinia, herpes simplex, fowlpox, and tracheo-laryngitis by the use of a slightly modified Gomori technic.¹⁰ While our evidence is not conclusive, it strongly favors the hypothesis of Hoagland, Ward, Smadel, and Rivers that the phosphatase activity of the inclusion body is due to tissue contamination.

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Comparative *in vitro* Resistance of Staphylococci to Penicillin and to Sodium Sulfathiazole.*†

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Investigations to be reported elsewhere¹ revealed that sulfonamide-resistant strains of staphylococcus were being recovered with increasing frequency at the University Hospitals, and that the majority of the resistant strains were being obtained from patients who had received sulfonamides prior to isolation of the organisms. In view of this, it appeared highly desirable to compare the *in vitro* resistance of a large number of strains of staphylococcus to the antistaphylococcic action of penicillin and to sodium sulfathiazole. Recent studies carried out with a relatively small number of cultures indicate that some strains of *Staphylococcus aureus* varied only slightly in their resistance to penicillin,² while other strains were quite insensitive.^{3,4,5,6}

* The penicillin was provided by the Office of Scientific Research and Development from supplies

Materials and Methods. Sixty-eight

assigned by the Committee on Medical Research for investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

† Aided by grants from the Committee on Scientific Research of the American Medical Association and from Sharp and Dohme, Inc.

¹ Spink, W. W., and Vivino, J. J., *J. Clin. Invest.*, in press.

² Rammelkamp, C. H., and Maxon, T., *PROC. SOC. EXP. BIOL. AND MED.*, 1942, **51**, 386.

³ Fleming, A., *Lancet*, 1942, **1**, 732.

⁴ Hobby, G. L., Meyer, K., and Chaffee, E., *PROC. SOC. EXP. BIOL. AND MED.*, 1942, **50**, 277.

⁵ Schnitzer, R. J., Camagni, L. J., and Buck, M., *PROC. SOC. EXP. BIOL. AND MED.*, 1943, **53**, 75.

⁶ Abraham, E. P., Chain, E., Fletcher, C. M., Gardener, A. D., Heatley, N. G., Jennings, M. H., and Florey, H. W., *Lancet*, 1941, **2**, 177.

TABLE I.
Effect of Size of Inoculum Upon Resistance of Staphylococci to Action of Penicillin.

Strain No.	No. of organisms	Penicillin (Oxford units per cc)									
		0.1	0.2	0.5	1.0	5.0	0.05	0.1	0.2	0.4	0.6
35	22,000,000	4+	4+	4+	4+	4+					
	220,000						4+	4+	4+	4+	3+
	22,000						4+	4+	4+	4+	0
	2,200						4+	4+	4+	3+	0
10	20,000,000	4+	4+	4+	4+	4+					
	200,000						4+	4+	4+	0	0
	20,000						4+	4+	4+	0	0
	2,000						4+	4+	4+	0	0
41	10,000,000	2+	+	0	0	0					
	100,000						1+	0	0	0	0
	10,000						2+	0	0	0	0
	1,000						3+	0	0	0	0
39	30,000,000	1+	0	0	0	0					
	300,000						1+	0	0	0	0
	30,000						0	0	0	0	0
	3,000						0	0	0	0	0
42	11,000,000	1+	1+	0	0	0					
	110,000						1+	0	0	0	0
	11,000						0	0	0	0	0
	1,100						1+	0	0	0	0

4+ = Maximum growth.

strains of coagulase-positive staphylococcus were investigated, all of which had been isolated from the lesions or body fluids of patients. A standard method was used for detecting the resistance of the strains to penicillin or to sodium sulfathiazole with the aid of a chemically-defined liquid medium, buffered at pH 7, in which the presence of a sulfonamide inhibitor was a negligible factor.¹ Just prior to carrying out a test, each strain was grown for several generations in the synthetic medium. A standard inoculum of staphylococci was used since it was observed that sulfonamide-resistance was a relative phenomenon, and the degree of resistance was directly related to the number of organisms employed in a test. On the contrary, it has been reported that the size of the inoculum was of minor importance in carrying out *in vitro* tests with penicillin.⁶

The *in vitro* tests were carried out by preparing 10-fold dilutions of a 24-hour culture in synthetic medium. Then 0.1 cc of the 10^{-3} dilution was added to each of a series of test tubes containing the medium. The approximate number of organisms in such an inocu-

lum was determined by making pour plates with the 10^{-7} dilution. Under these circumstances, there were between 100,000 and 300,000 organisms. Freshly prepared, aqueous solutions of sodium sulfathiazole were used for each test. Increasing concentrations of the sulfonamide, ranging from 0.5 mg per 100 cc up to 360 mg, were employed in the series of tubes containing the bacterial suspensions. Aqueous solutions of the sodium salt of penicillin from the same lot were freshly prepared and added to the bacterial suspensions with the final concentrations varying between 0.05 and 5 Oxford units per cubic centimeter. The final volume in all of the tubes was 10 cc. The bacterial suspensions with and without penicillin or sodium sulfathiazole were incubated for 48 hours at 37°C. Bacterial growth was quantitated on the basis of turbidity, and the inhibitory effect of penicillin or sodium sulfathiazole was determined by selecting that tube which revealed complete inhibition of growth in the presence of the lowest concentration of the antibacterial agent.

Results. The first series of observations were concerned with the effect of the size of

TABLE II.
Comparative *in vitro* Action of Penicillin and Sodium Sulfathiazole Against 68 Strains of Staphylococcus.

Strain No.	Penicillin inhibiting growth completely (Oxford units) per cc	Sodium sulfathiazole inhibiting growth completely (mg per 100 cc)	Strain No.	Penicillin inhibiting growth completely (Oxford units) per cc	Sodium sulfathiazole inhibiting growth completely (mg per 100 cc)
18	0.8	Less than 1	16	0.1	Less than 1
43	0.8	30	17	0.1	" " "
7	0.6	Less than 1	19	0.1	" " "
34	0.6	10	21	0.1	" " "
35	0.6	Less than 1	23	0.1	" " "
40	0.6	20	24	0.1	" " "
58	0.6	100	27	0.1	" " "
10	0.4	Less than 1	29	0.1	" " "
2	0.2	" " "	32	0.1	" " "
5	0.2	" " "	33	0.1	5
6	0.2	" " "	38	0.1	80
8	0.2	" " "	42	0.1	200
12	0.2	" " "	44	0.1	100
13	0.2	" " "	50	0.1	200
14	0.2	" " "	52	0.1	160
15	0.2	" " "	59	0.1	140
20	0.2	" " "	60	0.1	Less than 1
22	0.2	" " "	61	0.1	" " "
25	0.2	" " "	62	0.1	" " "
26	0.2	" " "	64	0.1	" " "
28	0.2	" " "	66	0.1	" " "
30	0.2	" " "	45	0.05	200
31	0.2	" " "	46	0.05	180
36	0.2	20	47	0.05	200
37	0.2	5	48	0.05	180
39	0.2	10	49	0.05	200
41	0.2	250	51	0.05	200
63	0.2	Less than 1	53	0.05	200
65	0.2	" " "	54	0.05	160
67	0.2	" " "	55	0.05	180
3	0.1	" " "	56	0.05	200
4	0.1	" " "	57	0.05	200
9	0.1	" " "	68	0.05	Less than 1
11	0.1	" " "			

the bacterial inoculum upon the inhibitory action of penicillin. For this purpose, 2 strains of staphylococcus relatively resistant to penicillin were selected, and compared with 3 strains considered to be sensitive to penicillin. The results are presented in Table I. The inhibitory effect of penicillin upon the growth of staphylococci sensitive to the action of the drug is but little affected by the number of cocci used in the test. This does not apply to the more resistant strains, and in such instances the interpretation of *in vitro* resistance studies might be confusing because of the number of bacteria utilized in a test.

The comparative results of the *in vitro* action of penicillin and sodium sulfathiazole upon 68 strains of staphylococcus are summarized in Table II. There appeared to be no relationship between the resistance of the cultures to penicillin and their resistance to sodium sulfathiazole, an observation which has been made by others with the pneumococcus.^{7,8} Strain 58 was highly resistant to

⁷ Powell, H. M., and Jameson, W. A., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 387.

⁸ McKee, C. M., and Rake, G., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 275.

the antibacterial action of both penicillin and sodium sulfathiazole. Strain 41 is the most resistant culture for the sulfonamides that has been encountered over a period of 3 years, and yet its growth was inhibited by 0.2 unit of penicillin.

Since several strains were found to be relatively resistant to penicillin but sensitive to sodium sulfathiazole, it appeared that simultaneous exposure of the organisms to both agents would require lower concentrations of each to inhibit growth completely than that found to be necessary when either penicillin or sodium sulfathiazole was used alone. Rammelkamp and Keefer⁹ observed that small amounts of penicillin without demonstrable inhibitory effect for staphylococci enhanced the antistaphylococcic action of sulfathiazole in defibrinated blood. Observations were made with strains 18, 34, 40, and 43, and in each instance it was observed that smaller concentrations of penicillin and sodium sulfathiazole were necessary to inhibit growth than was required when either agent was used alone.

Discussion and Conclusions. When 68 strains of pathogenic staphylococcus were subjected to the *in vitro* action of sodium penicillin, 8 of the strains, or approximately 12%, were found to be quite resistant, requiring from 0.4 units per cc to 0.8 units of penicillin before growth was completely inhibited. The

growth of the remaining 60 strains necessitated the following amounts of penicillin before growth was inhibited: 22 strains, or about 32%, required 0.2 units; 25 or approximately 37%, required 0.1 unit; while 12 or about 18%, failed to grow in the presence of 0.05 unit. About 28% of the strains were found to be highly resistant to the antistaphylococcic action of sodium sulfathiazole. There was no relationship between resistance to penicillin and to the sulfonamide. The evidence at hand would indicate that the mechanism whereby staphylococci become resistant to the sulfonamides is different and unrelated to the development of resistance to penicillin.¹⁰⁻¹⁴

Since some strains of staphylococcus isolated from patients appear to be naturally resistant to penicillin, and yet, sensitive to sodium sulfathiazole, it would not be unreasonable to assume that a combination of therapy with penicillin and sulfathiazole might be advantageous in the management of some types of staphylococcic sepsis. Further clinical observations are necessary to substantiate this, but preliminary investigations appear to bear out this assumption.

¹⁰ Landy, M., Larkum, N. W., Oswald, E. J., and Streightoff, F., *Science*, 1943, **97**, 265.

¹¹ Spink, W. W., Wright, L. D., Vivino, J. J., and Skeggs, H. R., *J. Exp. Med.*, in press.

¹² Gardner, A. D., *Nature*, 1940, **146**, 837.

¹³ Smith, L. D., and Hay, T., *J. Franklin Instit.*, 1942, **233**, 598.

¹⁴ Hobby, G. L., Meyer, K., and Chaffee, E., *Proc. Soc. Exp. Biol. and Med.*, 1942, **50**, 281.

⁹ Rammelkamp, C. H., and Keefer, C. S., *J. Clin. Invest.*, 1943, **22**, 649.

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Antibacterial Effect of Whole Blood upon Strains of Staphylococci Sensitive and Resistant to Penicillin.*†

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In a previous report,¹ it was shown that coagulase-positive strains of staphylococcus isolated from human subjects varied in their susceptibility to the *in vitro* action of penicillin. The purpose of the present investiga-

tions was to compare the antibacterial action of defibrinated human blood upon strains of staphylococcus adapted to grow in increasing concentrations of penicillin with the parent strains, whose growth was readily inhibited

by penicillin. Prior observations had revealed that normal human blood, with but few exceptions, possessed little or no killing effect for small numbers of pathogenic staphylococci.² In general, coagulase-positive strains of staphylococcus resisted the bactericidal action of human whole blood, whereas coagulase-negative strains were killed in large numbers.³

It has been reported that cultures of pneumococci, streptococci, and staphylococci may develop an increased resistance to penicillin following *in vitro* and *in vivo* exposure of the organisms to the drug.^{4,5,6,7}

Materials and Methods. Five strains of coagulase-positive staphylococci were utilized. The *in vitro* sensitivity of these strains for both penicillin and sodium sulfathiazole was first ascertained by the dilution method with a standardized inoculum of organisms in a synthetic medium.^{1,8} The Eden strain was isolated from the blood stream of a child, while Eden I was obtained from the exudate of an osteomyelitic sinus after this child had received large doses of penicillin and sulfathiazole. The Rutgers strain was cultured from the exudate present in an extensive sub-

cutaneous lesion of a child. The Ked and Nelson strains were obtained from draining osteomyelitic sinuses, present in each of 2 children. Of the five strains, Rutgers and Nelson were highly resistant to the *in vitro* action of sodium sulfathiazole.

The sensitivity of each strain for penicillin was first determined, and then resistance to the drug was developed in the following manner: each of several tubes contained 9 cc of the synthetic medium to which was added 1 cc of sodium penicillin dissolved in isotonic saline solution. A standard loopful of a 24- to 48-hour culture of organisms in synthetic medium was seeded to medium containing the added penicillin in a concentration which permitted demonstrable growth within 24 hours. Loopful transfers of the organisms were made every 24 to 48 hours in a given concentration of penicillin until maximum growth could be attained within 24 hours. Then the concentration of penicillin was increased and the serial transfers repeated. Simultaneously, serial transfers were made with the parent strain in 5 cc of synthetic medium. Comparative observations were also made to ascertain whether there was any difference in the rate of development of resistance in veal infusion broth and in synthetic medium. When the maximum degree of resistance to penicillin had been attained in the synthetic medium, the resistance of the strains to sodium sulfathiazole was compared with the parent strains.

The bactericidal action of human blood for the resistant strains and their susceptible parent strains was performed in the same manner as described elsewhere.³ Briefly, the method was as follows: fresh defibrinated blood from normal individuals was used. Ten-fold dilutions were prepared in broth from a 24-hour culture grown in synthetic medium, and 0.1 cc of each dilution (up to 10^{-7}) was added to 0.5 cc of the defibrinated blood in small pyrex test tubes. The approximate number of organisms seeded to each tube was determined by making pour plates with 0.1 cc of the 10^{-7} dilutions. The tubes were sealed in a gas-oxygen flame, and then rotated for 48 hours at 37°C in a box contained in an incubator. At the end of this time, the contents of each tube were cultured for viable

* The penicillin was provided by the Office of Scientific Research and Development from supplies assigned by the Committee on Medical Research for investigations recommended by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

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¹ Spink, W. W., Ferris, V., and Vivino, J. J., submitted for publication.

² Spink, W. W., and Paine, J. R., *J. Immunol.*, 1940, **38**, 383.

³ Spink, W. W., and Vivino, J. J., *J. Clin. Invest.*, 1942, **21**, 353.

⁴ Abraham, E. P., Chain, E., Fletcher, C. M., Gardiner, A. D., Heatley, N. G., Jennings, M. H., and Florey, H. W., *Lancet*, 1941, **2**, 177.

⁵ Rammelkamp, C. H., and Maxon, T., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 386.

⁶ McKee, C. M., and Houck, C. L., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 33.

⁷ Schmidt, L. H., and Sesler, C. L., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 353.

⁸ Spink, W. W., and Vivino, J. J., *J. Clin. Invest.*, in press.

organisms. In a second series of observations, the bactericidal action of whole blood with and without added sodium sulfathiazole was quantitated.

Results. Development of in vitro resistance to penicillin. Strains Eden and Ked were initially inhibited respectively by 0.04 and 0.08 Oxford units per cc of penicillin, but maximum growth took place in the presence of 0.01 units. The growth of both strains was inhibited by less than 1 mg per 100 cc of sodium sulfathiazole. After 109 serial transfers for each of the strains in increasing concentrations of penicillin maximum growth was obtained in 1 unit per cc. Both strains were then transferred to veal agar slants and placed in a refrigerator for 3 months. At the end of this period, the cultures were seeded to synthetic medium for 4 generations, and then exposed to penicillin. Both strains had retained their resistance to penicillin. After 101 additional transfers in increasing concentrations of penicillin, both strains were capable of growing in the presence of 50 units per cc of penicillin. Comparative studies with these 2 strains revealed that both could be more readily adapted to grow in the presence of increasing concentrations of penicillin when veal infusion broth was used as the medium instead of synthetic medium. Although both cultures developed a high degree of resistance to the antistaphylococcic action of penicillin, both remained sensitive to the inhibitory effect of sodium sulfathiazole. While the resistance of both of these strains to penicillin had been induced by growing the cultures in synthetic medium containing penicillin, it was observed that the cultures retained this resistance when veal infusion broth was used as the medium.

Studies were made with the Ked culture to determine if there was any difference in the rate at which this strain developed resistance to penicillin as compared to sodium sulfathiazole. Organisms were exposed simultaneously to increasing concentrations of each of the drugs in synthetic medium. Under these circumstances, Ked became more readily adapted to grow in the presence of the sulfonamides than in the penicillin.

The Rutgers and Nelson strains were both

highly resistant to sodium sulfathiazole, the former requiring a concentration of 160 mg per 100 cc before growth was completely inhibited, and the latter 180 mg. On the other hand, both cultures were quite sensitive to penicillin, the growth of Rutgers being inhibited by 0.06 Oxford units per cc, and Nelson by 0.05 units. After 94 serial transfers in the presence of increasing concentrations of penicillin, both cultures grew readily in 4 cc units per cc, with complete inhibition of growth taking place in 6 units per cc.

It had been previously reported that a strain of *Staphylococcus aureus* having acquired resistance to penicillin showed no fundamental change in biological or metabolic activities as compared to the parent strain, except that the rates at which these activities were carried out were slower for the resistant strain.⁴ These observations had been confirmed in the present study. The resistant strains retained their property of coagulating plasma. Acquired resistance to penicillin was associated with morphological changes of the bacterial cells, which appeared to be permanently acquired characteristics. The cells showed marked pleomorphism with large spherical cell bodies quite apparent. The parent cultures of Rutgers and Nelson had previously been shown to produce a brown-orange pigment in the presence of high concentrations of sodium sulfathiazole.⁹ While the cultures of Rutgers and Nelson adapted to resist penicillin still retained the same degree of resistance for sodium sulfathiazole as the parent strains, the pigment produced by the adapted strains was much less than that produced by the parent strains.

Development of in vivo resistance to penicillin and sodium sulfathiazole. The growth of Eden, originally isolated from the blood stream of a child, was inhibited by 0.04 Oxford unit per cc of penicillin and by less than 1 mg per 100 cc of sodium sulfathiazole. She was given penicillin parenterally and dramatic improvement followed. However, a complication of her illness was the development of osteomyelitis of the left femur which resulted in a progressive decalcification of the bone.

⁹ Spink, W. W., and Vivino, J. J., *Science*, 1943, **98**, 44.

TABLE I.

Antibacterial Action of Human Defibrinated Blood upon Strains of *Staphylococcus* Sensitive and Resistant to the Action of Penicillin.

Strain	Penicillin inhibiting growth completely (Oxford units per cc)	Sod. sulfathiazole inhibiting growth completely (mg per 100 cc)	Dilution of organisms							No. organisms 107 dilution
			101	102	103	104	105	106	107	
Eden (parent)	0.04	Less than 1	4+	4+	4+	4+	4+	4+	0	24
Eden (resistant)	80	" " "	3+	2+	2+	0	0	0	0	11
Ked (parent)	0.06	" " "	4+	4+	4+	4+	4+	4+	4+	19
Ked (resistant)	120	" " "	4+	4+	4+	4+	2+	0	0	13
Rutgers (parent)	0.06	160	4+	4+	4+	4+	4+	4+	2+	22
Rutgers (resistant)	6	160	4+	4+	+	+	0	0	0	20
Nelson (parent)	0.05	180	4+	4+	4+	4+	4+	4+	4+	12
Nelson (resistant)	6	180	4+	4+	4+	+	0	0	0	25
Eden (parent)	0.04	Less than 1	4+	4+	4+	3+	3+	0	0	25
Eden I (resistant)	0.8	200	4+	4+	3+	2+	0	0	0	6

0 = Growth.

4+ = Maximum growth.

Over a period of weeks she was given 225 g of sulfathiazole. Fourteen months after the onset of her illness, there was still active involvement of the bone, and she was given over two million Oxford units of penicillin. This resulted in improvement. A site over the infected bone became reddened, and finally spontaneous rupture and evacuation of purulent material occurred from which Eden I was cultured. The growth of Eden I was inhibited by 0.8 units of penicillin and 200 mg of sodium sulfathiazole. It was concluded that the resistance of Eden I was induced by the administration of large amounts of penicillin and sodium sulfathiazole.

Bactericidal Tests. The killing effect of whole defibrinated blood upon the 5 parent strains and their subcultures, which had developed a resistance to penicillin, is summarized in Table I. In every instance except in the case of Eden I, the resistant cultures were killed in larger numbers than were the sensitive strains. The degree of resistance to penicillin manifested by Eden I was much less than that shown for the other resistant cultures. The ability of the organisms to grow in the presence of sodium sulfathiazole was unrelated to the bactericidal action of the blood. The important difference in the anti-

staphylococcic action of the blood appeared to be related to the resistance of the cultures to penicillin. Strains resistant to the sulfonamide were as capable of growing in defibrinated blood as their nonresistant progenitors.

When the same experiments were repeated with and without added sodium sulfathiazole, it was observed that the sulfonamide exerted an additional bactericidal effect provided the strain being tested was sensitive to the action of sodium sulfathiazole.

Discussion and Conclusions. Strains of staphylococcus adapted to grow in the presence of the sulfonamides are just as invasive as other strains which are inhibited in growth by the sulfonamides. Whole, human blood has no greater bactericidal action on the sulfonamide-resistant strains than on the sulfonamide-sensitive strains. Strains of staphylococcus adapt themselves to grow in the presence of penicillin at a slower rate and to a lesser degree than is apparent for the sulfonamides. And probably of considerable clinical importance is that an increased resistance to penicillin is accompanied by the development of strains which are more susceptible to the bactericidal action of whole blood, and possibly to the other defense mechanisms of the host.

Experimental Diabetes Produced by Alloxan.

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Experimental selective necrosis of the islands of Langerhans has recently been recorded by Dunn, Sheehan, and McLetchie.¹ In a limited number of animals the injection of alloxan produced a selective necrosis of islet tissue and the epithelium of the convoluted tubules with characteristic physiological symptoms. These observations were confirmed and extended to dogs and other species including man.^{2,3,4} The obvious value of an experimentally produced diabetes and a search for the mechanism of action of alloxan led us to the following studies.

Influence on Blood-Sugar Level. Rabbit. Twenty-five animals were used for studies on the influence of alloxan on blood-sugar level. A dose ranging from 10 mg per kg to 300 mg per kg intravenously in a 5% solution was administered and blood samples taken for analysis at intervals thereafter.⁵ The compound was well tolerated and even in large doses no untoward symptoms were observed for a period of 5 hours. Both fasted and unfasted animals were used. The data of these experiments are summarized in Table I.

An analysis of the data in Table I reveals that these animals group themselves into 2 classes. With small doses up to 50 mg per kg there is little or no influence upon blood-sugar level. Larger doses appear to produce in most animals an immediate rise in blood sugar to concentrations of 200 mg % or greater followed by a subsequent return to, or below the normal value within 24 hours and this, in turn, is followed by a secondary rise to diabetic level, which is maintained for weeks. An ideal curve is given in Fig. 1. Jacobs⁶ previously observed the initial rise in blood sugar concentrations and the subsequent fall to low levels but did not extend his observations beyond 24 hours, hence, the secondary rise was not observed. Not all animals injected respond uniformly. Our observation is that if a diabetic response is to be obtained in an animal, the initial curve produced within the first 24 hours will demonstrate this fact. A refractory animal generally will not respond to successive doses of alloxan.

In order to determine the source of the elevated blood glucose liver glycogen deter-

TABLE I.
Influence of Alloxan on Blood-Sugar Level of Rabbit.

No. of animals	Dosage, mg/kg	Response	Blood sugar mg per 100 cc blood		
			Control, avg	2-4 hr after inj.	Daily avg thereafter
2	10	Negative	127	134	102
3	25	"	116	118	115
5	50	1—diabetic	106*	259*	268*
13	100	8—diabetic	109*	267*	295*
				(185-400)	(247-396)
2	300	Hypoglycemic convulsions	107	39	death in 5 hr

* Average values for diabetic animals only.

¹ Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **11**, 384.

² Gomori, G., and Goldner, M. G., *PROC. SOC. EXP. BIOL. AND MED.*, 1943, **54**, 287.

³ Bailey, C. C., and Bailey, O. T., *J. A. M. A.*, 1943, **122**, 1165.

⁴ Brunshwig, A., and Allen, J. G., *Cancer Research*, 1944, **4**, 45.

⁵ Folin, O., *J. Biol. Chem.*, 1928, **77**, 421.

⁶ Jacobs, H. R., *PROC. SOC. EXP. BIOL. AND MED.*, 1937-38, **37**, 407.

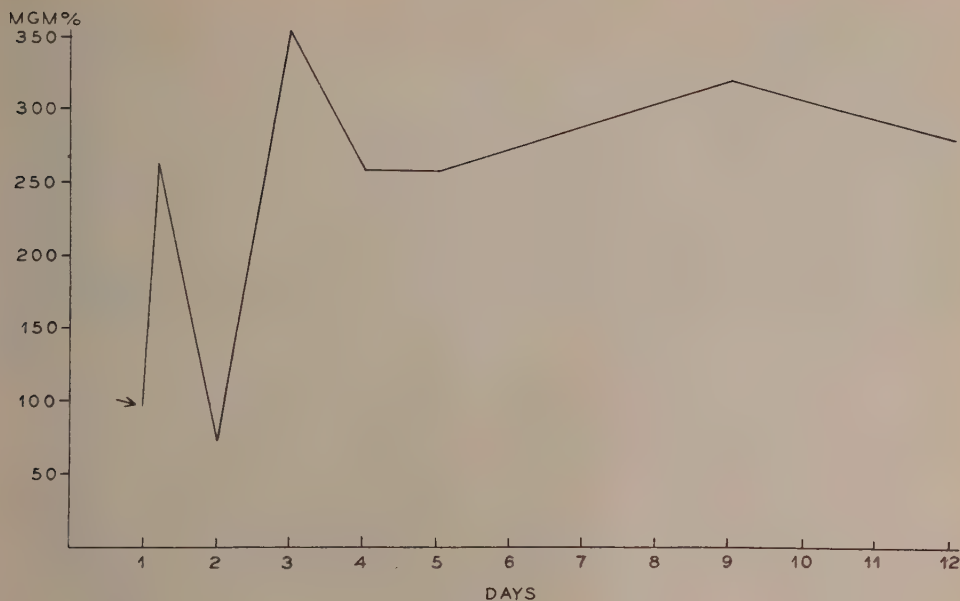


FIG. 1.

A typical blood-sugar curve obtained after intravenous injection (arrow) of 100 mg per kg of alloxan.

minations were made on 7 rabbits in periods varying from 3 hours to 25 days following injection. All values were well within a normal range, averaging 3.46%. A similar result was observed in 10 rats sacrificed 48 hours following injection of 200 mg per kilo dosages of alloxan. This type of evidence suggests that the capacity of the liver to store carbohydrate as glycogen is undiminished, but does not satisfy the question of elevated blood sugar. However, the initial rise in blood glucose (Fig. 1) may be due to adrenal stimulation as is indicated by typical epinephrine type blood pressure curves obtained from dogs after 100 mg doses of alloxan, and from the histological findings.

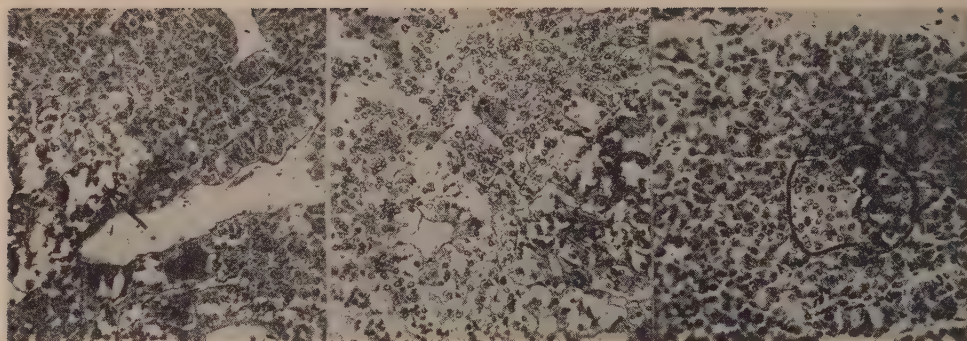
It may be noted that the extreme fatty infiltration reported for the diabetic dog⁷ has not been evidenced in our rabbits. Values of 12.4, 11.2, and 13.1 g % dry weight have been obtained in animals diabetic for 7, 11, and 25 days respectively.

Histological Findings. Autopsies were performed on 8 animals under nembutal or dial

anesthesia. Tissues from the pancreas, liver, adrenals, and kidneys were appropriately fixed for cytological study. In gross appearance all organs were normal in these animals. Routine hematoxylin-eosin and Heidenhain-azan stains were used but, in addition, sections of the pancreas were treated with Gomori's modification of the azan technic and Bensley's neutral gentian for demonstration of the alpha and beta cells.

Our histological observations on the effect of acutely toxic doses of alloxan on the pancreas are essentially similar to those previously described for the rabbit,¹ dog,^{4,7} and rat.² Changes noted are first, a pyknosis of the beta cells with the alpha cells unaffected, and, secondly, a shrinkage of the cells accompanied by degranulation. No histological changes were noted in the kidney or liver of these animals. A marked effect, however, was seen in the adrenal gland. In non-localized areas of the medulla there occurred a marked fragmentation and shrinkage of the cells with some cellular debris resulting (Fig. 2). Remaining areas of the gland appear to be unaffected but no definite statement can

⁷ Goldner, M. G., and Gomori, G., *Endocrinology*, 1943, **33**, 297.



A

B

C

FIG. 2.

A. Adrenal medulla, rabbit, 300 mg per kilo. Fragmentation and shrinkage of cell cords in certain areas (arrow) with nuclear remnants. Adjacent areas show normal cellular arrangement. Heidenhain-azan $\times 100$.

B. Adrenal medulla, rabbit, diabetic 7 days. Small areas show some fragmentation and shrinkage. In less affected glands vacuolation is the only noticeable effect. Hematoxylin-eosin $\times 100$.

C. Pancreatic islet, rabbit, diabetic 25 days. The outlined islet is composed of the deeply stained alpha cells and nongranular cells. $\times 100$.

be made with respect to a variation in the chromaffin reaction.

Microscopical findings on diabetic animals sacrificed on the 7th, 11th, and 25th days after alloxan administration show significant effects in only the pancreas and adrenal glands. The pancreas of the animals diabetic for 7 and 11 days are essentially alike. When specially stained they show apparently a normal quota of islets but many of them are small and made up entirely of granular alpha cells. In a few islets undifferentiated cells appear which are practically devoid of cytoplasm. Their nuclei, conspicuous by the reduction in cytoplasm, are normal in reaction. In the 25-day diabetic animal no pathology could be detected in hematoxylin-eosin preparations; the islets are numerous, many of normal size distribution and cellular content. Specially stained preparations show the majority of cells either alpha or nongranular (Fig. 2) but with some islets showing granular beta cells. The granular content of these cells varies considerably ranging from the normal distribution to cells with very few granules. In many islets the ratio between the alpha and nongranular cells is equal to that normally occurring between the alpha and beta cells. Small islets are composed entirely

of either alpha or nongranular cells. There is a suggestion, therefore, that with the dosages employed complete beta cell necrosis does not occur but that regeneration of a functional cell is possible with an expected amelioration of the diabetes.

The adrenal medulla of the permanently diabetic animals are normal excepting for small areas in which the cells show some degree of cytoplasmic vacuolation (Fig. 2). This is neither as extensive nor exhibits the degree of shrinkage and fragmentation seen in the animals given acutely toxic doses. Absence of any medullary damage in a 25-day diabetic animal suggests that a complete recovery has ensued by this period.

Summary. The administration of alloxan in one suitable dose produces a pancreatic experimental diabetes. Satisfactory animals exhibit a prolonged high blood-sugar level that is maintained for weeks. No renal lesions are observed in such animals and the fat and glycogen content of the liver is within normal limits. Adrenal and pancreatic islet cellular changes may explain the character of the acute phase.

The expense of this investigation has been defrayed in part by a grant from the Bressler Research Alumni Fund of the University of Maryland.

Two Characteristic and Sensitive Color Reactions Between Sulfhydryl Compounds and Thymonucleic Acid.*

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Thymonucleic acid reacts with cysteine in H_2SO_4 at 40° in two different ways depending upon which of the two reactants is in excess. The reaction products are colored and can be used for the detection of cysteine in a dilution of $1:10^6$ and of thymonucleic acid in $1:10^4$. Glutathione also gives a positive reaction in a dilution of $1:300,000$. The procedure for the two reactions is as follows:

I. To 1 cc of a solution of Na thymonucleate (0.01 to 0.1%) are added, with cooling, 0.04 cc of a 5% solution of cysteine hydrochloride and 5 cc of a mixture of 190 cc of H_2O and 450 cc of concentrated H_2SO_4 . The mixture is put into a water bath at 40° for 5 minutes. A deep pink color appears, which is stable for hours. It shows a sharp absorption maximum at $500\text{ m}\mu$. Cystine, homocystine, methionine, and dithiodiglycolic acid do not react even in high concentrations. Glutathione reacts like cysteine in equivalent concentrations. Thioglycolic acid reacts also, though a little less strongly than cysteine. Propyl mercaptan also gives the reactions, but pyruvic acid and acetone do not. When the desoxyribose is destroyed by heating the thymonucleic acid in 2% H_2SO_4 for 2 hours at 100° , the reaction with cysteine disappears completely. All these facts indicate that the colored substance is the product of a reaction between the desoxyribose of the thymonucleic acid and the sulfhydryl group of the cysteine (glutathione, thioglycolic acid).

The reaction can be used for the detection and probably for the colorimetric determination of small amounts of thymonucleic acid. In the range of 0.02-0.08% of thymonucleic acid the color intensity in white light is proportional to the concentration. The specificity

and accuracy of the reaction are about the same as that of the diphenylamine H_2SO_4 reaction. The chief advantage which the new reaction seems to possess is the possibility of use even in the presence of large amounts of proteins. This is not feasible with the diphenylamine reaction because proteins and products of their hydrolysis are precipitated by the weak acid used in that reaction. On the other hand, they dissolve easily in the much more concentrated acid used in the reaction with cysteine. However, it must be noted that in the presence of large amounts of proteins the color which develops is not pink but red-brown.

II. The character of the reaction between cysteine and thymonucleic acid changes completely when performed in the presence of a large excess of the latter acid. When 0.8 cc of a 0.002-0.01% solution of cysteine hydrochloride is mixed with 0.2 cc of a 1% solution of Na thymonucleinate, 5 cc of the H_2SO_4 water mixture as above are added, and the mixture held for 3 minutes at 40° , a red-brown color appears with a sharp absorption maximum at $410\text{ m}\mu$. With thioglycolic acid the sensitivity of the reaction is about the same as with cysteine. Glutathione shows the same red-brown color only in higher concentration (0.05%); in lower concentrations, down to 0.005%, the color developed is brown. But even in these lower concentrations the characteristic absorption maximum is at $410\text{ m}\mu$. The brown color cannot be regarded as characteristic, because other substances, like cystine at 0.2%, pyruvic acid at 1%, and even thymonucleic acid alone at higher concentration, show a yellow color, but it seems possible that the absorption maximum at $410\text{ m}\mu$ will prove characteristic, for the yellow reaction products formed with cystine and pyruvic acid do not show this maximum. The

* This work was carried out with the aid of a grant from the International Cancer Research Foundation.

reaction between cysteine and glutathione (but not thioglycolic acid) and thymonucleic acid; can be made about 10 times more sensitive if it is performed in the following way:

To 1 cc of a solution containing cysteine (glutathione) are added, with cooling, 1.7 cc of concentrated H_2SO_4 . The well-cooled mixture is then treated with 0.03 cc of a 1% solution of Na thymonucleinate and held at 40° for 3 minutes. The red-brown color with the same absorption maximum appears and can easily be recognized when the concentra-

tion of cysteine is only 0.0002%, or that of glutathione is 0.0005%.

Both color reactions of the thymonucleic acid with cysteine are probably due to the formation of some furfuryl compound from the desoxyribose. In fact, furfuraldehyde reacts with cysteine under the same conditions as thymonucleic acid forming a violet red product in presence of an excess of cysteine, and a brown color when the aldehyde is in excess. The latter reaction is much less sensitive than the reaction with thymonucleic acid.

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Experimental Production of Diabetic Cataract in the Rat.

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Two groups of workers describe the appearance of cataracts in animals with experimental diabetes. Dohan, Fish, and Lukens¹ observed this phenomenon after 5 months in a dog with permanent diabetes produced by injection of anterior pituitary lobe extract. Chaikoff and Lachman^{2,3} observed it after a year in 8 dogs without pancreas, which were kept alive with insulin and an adequate diet. The spontaneous appearance of cataracts in pancreatectomized rats, with progressive evolution to complete opacity of the lens, led us to investigate factors that might be involved.⁴

Technic and Methods. Male white rats were almost completely pancreatectomized when they weighed 60-80 g. A portion of pancreas was left which varied between 5% to 20% of the weight of the organ. The diet was the same as that given to all the other rats of the Institute, in which no evidence of cataract was detected.

After 7 hours fasting, blood sugar was determined with blood from the tail, following Hagedorn and Jensen's method. The lens was examined through an electric ophthalmoscope after dilatation of the pupil by atropin. A Gullstrand's lamp was used for more minute studies. We classified the lens lesions in 4 degrees of increasing severity, as follows: Type A, alteration of the anterior capsule of the lens (rugosity); Type B, opacity of the lens sutures; Type C, partial opacity of the lens parenchyma, and Type D, total opacity of the lens parenchyma.

The lens and blood sugar in rats were observed under 3 different experimental groups: (1) with 5% of pancreas; (2) with 20% of pancreas; (3) normal, without operation. A fourth group was specially formed with some animals in order to follow the evolution of blood sugar and lens lesions in them.

Results. (1) Rats with only 5% of pancreas: 41 rats were observed. The lesions of the lens appear 50 days after the operation and, generally speaking, get worse as time goes on (Table I). Lesions of Types C and D are rarer because the most seriously affected animals die soon, thus giving no time for the formation of the cataract. The frequency and severity of the lens lesions increase in direct

¹ Dohan, F., Fish, C., and Lukens, F., *Endocr.*, 1941, **28**, 341.

² Chaikoff, I. L., *PROC. SOC. EXP. BIOL. AND MED.*, 1935, **33**, 211.

³ Chaikoff, I. L., and Lachman, G. S., *PROC. SOC. EXP. BIOL. AND MED.*, 1933, **31**, 237.

⁴ Cramer, F. K., and Foglia, V. G., *Rev. Soc. Argent. Biol.*, 1943, **19**.

TABLE I.
Frequency and Types of Cataracts in 47 Pancreatectomized Rats from the Date of Operation.

Days of operation	0.50	50-125	125-200	200-275	275-325	325-400
With 5% of pancreas.						
Without lesions	10	—	—	—	—	—
Lesion Type A	—	4	5	2	—	—
" " B	—	3	4	2	2	—
" " C	—	—	1	2	1	—
" " D	—	—	2	1	1	1
Total	10	7	12	7	4	1
With 20% of pancreas.						
Without lesions	—	—	—	—	—	2
Lesion Type A	—	—	—	1	—	1
" " B	—	—	—	—	1	1
Total	—	—	—	1	1	4

relation with the level of blood sugar (Table II). Thus very few rats with a normal level of blood sugar have lesions, while all rats with a level greater than 220 mg % have lesions of the most serious types, C or D. The evolutionary character of the cataract is well marked. We have been able to follow the development of each type of lesion in the same rat. Lesion A takes 50 days to arise, but lesion D takes over 4 months and with very high levels of blood sugar (greater than 220 mg per 100 cc). Histological study of cataracts of Type D showed deep alterations in the cortical zone of the lens and very few if any in the capsular and nuclear zone.

TABLE II.
Frequency and Severity of Cataracts in 41 Pancreatectomized Rats According to the Blood Sugar Level.

Blood sugar mg %	80-120	120-220	More than 220
With 5% of pancreas.			
Without lesions	9	1	—
Lesion Type A	1	7	3
" " B	1	7	3
" " C	—	1	3
" " D	—	—	5
Total	11	16	14
With 20% of pancreas.			
Without lesions	2	—	—
Lesion Type A	1	1	1
" " B	—	1	—
Total	3	2	1

(2) Rats with 20% of pancreas: These show less incidence of diabetes than rats with 5% of pancreas. They also show less frequency and severity of lesions of the lens than those of the previous group (Tables I and II). The onset of the lesions took place after 200 days. Lesions were never more serious than those of Type B and could be observed only in rats with increased blood sugar.

(3) Normal rats: 100 rats were observed. None showed lesions of any type (B, C, or D). A few had lesions resembling Type A though not exactly like it. We did not find any lesions, either, in 80 rats with experimental arterial hypertension due to a renal cause.

Summary and Conclusions. In all rats with 5% of pancreas remaining after partial pancreatectomy, cataracts appear after a period of time which in the cases we observed was from 50 days onward. This cataract is progressive and its degree is in direct relation to the level of fasting blood sugar. Microscopic examination shows well marked lesions in the lens.

In rats with 20% of pancreas, the lens lesions take over 200 days to appear and are less serious in direct relation to the less serious character of the diabetes.

We have not observed any cataracts like these in normal rats.

Action of a Sulfonated Succinic Acid on the Respiratory Metabolism of Frog Tissues.*

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In the succinic acid cycle outlined by Szent-Gyorgyi¹ succinic acid serves in a catalytic capacity as a carrier between metabolites and the cytochrome - cytochromeoxidase - oxygen complex. In this scheme succinic acid is oxidized to fumaric acid. This reaction is catalyzed by succinic dehydrogenase and is reversible. Thus, the action of succinate serves the dual role of substrate and reversible catalyst-carrier in the respiratory enzyme system.

From a series of sulfonated succinic acid derivatives, it was considered of theoretic interest to investigate the action of potassium-sulfon-succinic acid. This compound contains a ($\text{SO}_2 \cdot \text{OK}$) group replacing one of the hydrogen atoms of the ethane residue.

A series of experiments on frog skin and muscle were carried out using the Warburg manometric technic. The tissues were suspended in Ringer-Phosphate, pH 7.5. Succinic acid and potassium-sulfon-succinic acid were neutralized with sodium hydroxide before use.

The oxygen consumption of frog skin was not noticeably influenced by the presence of 0.1 mg glucose or 0.04 mg succinate, the respiratory rate diminished with time (10 to 20% in 2 hours). The addition of 0.04 mg potassium-sulfon-succinate inhibited oxygen

consumption 20 to 40%.

The respiratory rate of sliced and minced muscle falls off rapidly with time, decreasing as much as 80% in 2 hours. The addition of 0.1 mg glucose did not arrest this fall. However, 0.04 mg succinate had a marked effect, increasing oxygen consumption 100% or more and maintaining this increased rate for several hours.

In contrast, the addition of 0.04 mg of potassium-sulfon-succinate to muscle resulted in a 25 to 30% depression of oxygen consumption compared to untreated controls to which no succinate had been added.

The addition of succinate in catalytic amounts (40 γ) increased the oxygen consumption of muscle by 30 to 40% and also maintained this rate for more than an hour. Upon the addition of 0.04 mg potassium-sulfon-succinate to the tissue, the oxygen uptake of which was accelerated by succinate, there resulted a 75% diminution of the oxygen uptake.

Conclusion. From these observations, it would appear that potassium-sulfon-succinate is not utilized as a substrate nor acts as a reversible carrier. It is capable, however, of inhibiting the catalytic activity of succinate when the latter is present in minute quantities.

This latter inhibition presents an analogy to the activity of para-aminobenzoic acid and the sulfa drugs. Owing to the widespread distribution of succinate in tissues and bacteria, the possible bacteriostatic action is being investigated.

* We are indebted to Dr. L. A. Sweet of the Laboratories of Parke, Davis and Co., Detroit, Michigan, for the preparation of potassium-sulfon-succinic acid.

¹ Szent-Gyorgyi, A., *Z. f. Physiol. Chem.*, 1935, **236**, 1.

Toxicity of Thiouracil in Normal and Thyroidectomized Rats.

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The mode of action of the group of anti-thyroid substances investigated by Mackenzie¹ and Astwood and his collaborators² consists, according to these authors, in an inhibition of formation of the metabolic hormone of the thyroid. It seems, however, that one possible explanation was not completely excluded, namely, that the thyroid hormone was consumed in the process of detoxifying, metabolizing the sulfur compounds, and thus was no longer available for maintenance of the metabolic rate of the animal. This theory was supported by Astwood's³ observation that the most active antithyroid compounds were of the lowest toxicity, which admits of the interpretation that the toxicity of some substances was due to the resistance to destruction by the effect of thyroid hormone. If the mechanism of action were according to this hypothesis, the toxicity of a compound should be higher in the thyroidectomized rat, where no thyroid hormone is available, than in the normal rat. Since our laboratory has always a large number of thyroidectomized rats on hand, we decided to determine the 50% lethal dose of thiouracil on both types of animals.

Method. The intake of a definite quantity of the toxic substance is essential for comparative tests, and, therefore, accurately measured amounts were administered by stomach tube. To dissolve the thiouracil in the drinking water or mix it with the food is less suitable for toxicity experiments. Even if the drinking fountain does not drip when touched by the rat's mouth and thus spill part of the water, and if spillage of food can be avoided absolutely, there is still the diffi-

culty that the quantity of ingestion varies with the rat's desire for food and drink.

A difficulty was encountered in the low solubility of thiouracil in water as compared with the dose necessary to cause toxic symptoms. The substance had to be given in the form of an aqueous suspension, in order to administer sufficient quantities in a volume of liquid not damaging to the animal's stomach. Astwood (*l.c.*) reported that one gram per kilo body weight daily had to be exceeded to cause toxic symptoms. Although in our experience the innocuousness of the substance was not quite that high, it was soon evident that the drug had to be given in amounts so large as to preclude the possibility of complete absorption and so as to appear in the stool, before any signs of poisoning were noted. In view of the uncertain ratio between the dose administered and portion absorbed, the 50% MLD became quite meaningless.

There was no difference between the normal and thyroidectomized animals in their reaction to the treatment. The results are condensed in Table I without keeping the two groups separated. The first symptoms of beginning intoxication were a reduction in food intake and roughness of the fur. Subsequently, most

TABLE I.

No. of rats	Daily dosage, mg/kg wt	Length of treatment in days	Pathological changes
1	0.25	10 K	None
3	0.25	10-15 K	Mild
3	0.50	15-22 K	None
2	0.50	6; 7 D	Severe
3	0.50	9-16 K	Moderate
4	1.00	3-5 D	Severe
4	2.00	3 D	"
2	2.00	2 K	"
4	3.00	2-5 D	"
4	4.00	1-4 D	"

All animals were males varying in weight from 150-231 g with an average of 200. In column "Length of treatment" D means died, K means killed.

¹ Mackenzie, C. G., and Mackenzie, J. B., *Endocrinology*, 1943, **32**, 185.

² Astwood, E. B., Sullivan, J., Bissell, A., and Tyslowitz, R., *Ibid.*, 1943, **32**, 210.

³ Astwood, E. B., *J. Pharm. and Exp. Therap.*, 1943, **78**, 88.

animals developed bloody urine, some also bled from the nose and had diarrhea, and a large number showed tremor. At autopsy the first impression was always the more or less generalized congestion of all internal organs and the whiteness of the intestines caused by the diffuse filling with the thiouracil. This latter was occasionally obliterated in part by intestinal hemorrhage. Lung edema, as observed by Mackenzie and Mackenzie⁴ after thiourea treatment occurred occasionally but

⁴ Mackenzie, J. B., and Mackenzie, C. G., *Proc. Soc. Exp. Biol. and Med.*, 1943, **54**, 34.

was not always the first pathological change. When the lungs were congested, hemorrhage was usually absent. The most damaged organs were, first, the kidneys and, secondly, the liver; occasionally the spleen showed signs of damage, also.

Conclusion. Thiouracil is relatively non-toxic. Continued large doses cause cachexia, tremor, convulsions, bloody urine and feces. Most internal organs show hemorrhagic congestion; kidneys and liver seem to be damaged invariably; edema of the lungs is often, but not always, present.

14529

Increased Growth Produced by Gelatin in Trout Fed Meatless Diets.*

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For many years research on the nutrition of fish has indicated that trout require fresh meat in their diet for normal growth and maintenance.^{1,2,3} The more recent work of McCay and his collaborators points to the existence of an indispensable labile factor present in fresh meat which has been termed "Factor H."⁴ It has been demonstrated that the latter is required for growth and normal erythrocyte production in trout.

Restrictions in the use of meat as an animal feed during the present war food emergency threaten to restrict seriously the culture of trout in hatcheries throughout this country.

This situation prompted a search for satisfactory substitutes for meat in fish diets.

Methods. All experiments were carried out in the Biological Laboratories of the James Nevin State Hatchery,[‡] Madison. Healthy rainbow trout, *Salmo gairdnerii irideus*, were obtained directly from the stock raceways of the hatchery. The hatchery stock diet, consisting of ground fresh liver 47.5, ground carp 47.5, and dried brewer's yeast 5, was fed prior to the beginning of the experiment.

The procedure consisted of feeding the same experimental diet to 2 tanks of fish once daily, 6 days a week. An equal number of fish, from the same lot and chosen for their uniformity, were placed into one tank; 300 or more fish per tank were always used. Average weights were obtained once a week by transferring all the fish in a tank to a weighed quantity of water, and dividing the resultant fish weight by the total number of surviving fish.

Of an extensive series of diets which have

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station and supported through special grants from the Graduate School of the University.

[†] Biologist, Wisconsin Conservation Department.

¹ Davis, H. S., and Lord, R. F., *U. S. Dept. Comm., Bur. of Fish, Doc. No. 1079*, 1930, 130.

² McCay, C. H., *Ann. Rev. Biochem.*, 1937, **6**, 453.

³ Tunison, A. V., Brockway, D. R., Maxwell, J. M., Dorr, A. L., and McCay, C. M., *Cortland Hatchery Report*, No. 11, N. Y. State Conserv. Dept., Albany, New York.

⁴ McCay, C. M., *Science*, 1928, **67**, 249.

[‡] This project was made possible by the cooperation of the Biology Division of the Wisconsin Conservation Department and its Chief, Dr. Edward Schneberger.

been devised and tested, the following have been selected to illustrate the effect obtained with gelatin. The experimental basal ration consisted of skim milk powder 25, cottonseed oil meal 15, alfalfa leaf meal 10, cod liver oil 2, dried brewer's yeast 3, iodized salt 0.5, yellow corn meal 41.5, and *l*-ascorbic acid 4.54 g per 100 lbs. of ration. Various dried liver preparations[§] were included at a level of 3%. The control meat diet was composed of the hatchery stock mixture of fresh liver, carp, and yeast.

Experimental. The basal ration supplemented with 1:20 liver powder, liver fraction "L" or fraction "M" were fed in unrestricted quantities for a period of 9 weeks. During this entire period the weight of the fish receiving all experimental diets was essentially unchanged from their initial weight. However, the fish receiving the control meat diet, alone, showed a gain of 67% above the starting weight during the same period.

Effect of force feeding basal rations in gelatin capsules. Since a large residue was always present after feeding the experimental rations in the usual way, it was suggested that a factor in their failure to induce growth might be one of palatability. Thus, to ensure a satisfactory test of the nutritional value of these diets, they were enclosed in gelatin capsules and hand-fed to the trout.^{||} Sufficient diet was forced into the capsules so that each contained 1.5% (diet weighed in dry state) of the average body weight of the fish exclusive of the weight of the gelatin. The latter was estimated to be about 0.3% of the body weight. It was impossible to enclose the meat control diet in capsules. However, when this diet was fed to fish, it was consumed immediately and rarely was any residue seen. Therefore, 15 parts of melted gelatin was mixed with 85 parts of the meat diet and this was fed at an intake calculated at 1.8% (diet weighed in a wet state) of the total weight of the fish. daily.

At the end of 7 weeks of feeding, the aver-

age weight of the trout receiving the capsule-contained meatless ration had increased from 40.0 g to 50.2 g (trout larger than those used in *ad libitum* feeding experiments were required for the feeding of capsules), while the weight of the fish receiving the meat plus gelatin diet had increased to 43.8 g from a starting weight of 40.0 g. It would appear that the restricted meat intake of the latter group, which was calculated on a wet basis, furnishes an explanation for the sub-optimum rate of growth.

Effect of feeding of basal rations supplemented with gelatin and factors affecting palatability. The basal rations were supplemented in the following ways: plus 15% ground gelatin; plus 20% fresh ground spleen; plus 20% "stick";[¶] plus anise oil (sufficient to give a distinct licorice flavor). For the first 4 weeks of the experiment, the rations were fed at an intake calculated at 5% of the total weight of fish in the group. Subsequent to the fourth week the level was increased to 10%. The group fed the original basal ration gained only slightly, anise oil did not increase the response, supplementing the ration with spleen or "stick" produced a slow uniform gain in weight throughout the course of the experiment. Although the fish fed the ration supplemented with gelatin lost some weight the first week, as they became accustomed to the new diet, they grew as rapidly as the group receiving the control meat diet (Fig. 1). It is noteworthy that the added gelatin serves only as a stimulus to initial growth with the meatless diets used for fish receiving these supplemented rations begin to lose weight rapidly after approximately 7 weeks (Fig. 1). It would appear that the failure to sustain growth rests in further deficiencies in the diets, one of which may be that of Factor H. In contradiction to an earlier report,⁵ the Cornell workers re-

[¶] "Stick" is a concentrated residue obtained from the aqueous liquor in which processed meats have been steeped or cooked. It was supplied by Oscar Mayer and Co., Madison.

[§] We are indebted to Dr. David Klein of the Wilson Laboratories, Chicago, for generous supplies of the liver fractions.

^{||} The procedure of the hand-feeding technic as applied to controlled nutritional studies in fish will be more fully discussed elsewhere.

⁵ Phillips, A. M., Tunison, A. V., Fenn, A. H., Mitchell, C. R., and McCay, C. M., *Cortland Hatchery Report*, No. 9, N. Y. State Conserv. Dept., Albany, New York.

FIGURE 1. THE INCREASED GROWTH INDUCED IN TROUT FED A MEATLESS DIET SUPPLEMENTED WITH GELATIN

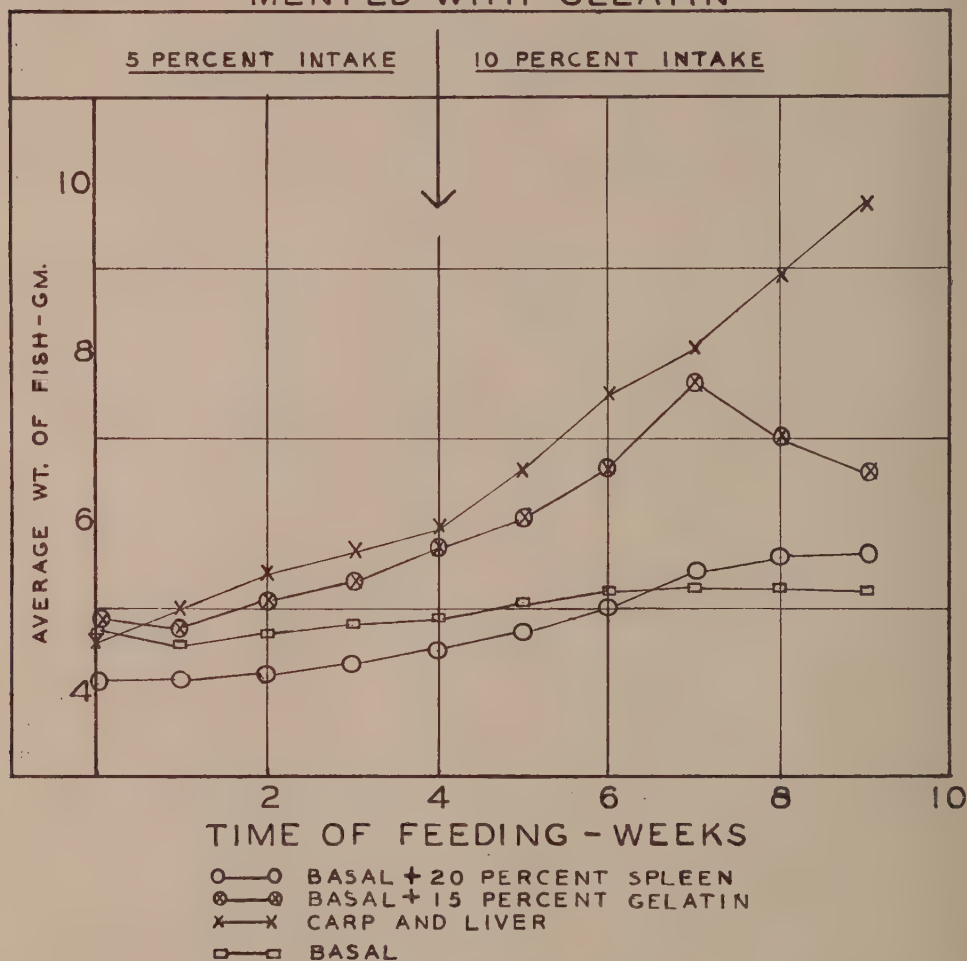


FIG. 1.

cently stated that dried liver preparations are devoid of anti-anemic properties.³

Discussion. The increased requirement of the chick for arginine and glycine for the production of muscle creatine and feathers⁶ suggests that an analogous situation may exist in the trout. It has been found that gelatin satisfactorily supplies these necessary amino

acids in the nutrition of the chick.⁷ Walters⁸ states that fish scales and bird feathers may have a homologous origin. Thus, it can be suggested that formation of the epithelial surface and dermal scales of the trout provides a nutritional demand similar to that in the

⁷ Briggs, G. M., Jr., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1942, **144**, 47.

⁶ Hegsted, D. M., Briggs, G. M., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **140**, 191.

⁸ Walter, H. E., *Biology of the Vertebrates*, New York, 1939, p. 247.

chick.**

Summary. It has been shown that growing

** The growth promoting property of the gelatin-supplemented diets has been substantiated in practical feeding tests carried out at the Westfield (Wis.) State Hatchery. Brook trout (*Salvelinus fontinalis*) fingerlings were fed either the experimental diet or the hatchery stock diet of pork spleen mixed occasionally with brewer's yeast at 10% of the body weight. After 8 weeks the fish receiving the meat diet had gained 52% over the initial weight while those receiving the experimental diet had gained 40%.

trout require a diet containing fresh meat. Mixed diets devoid of fresh meat did not produce any increase in the growth of trout. Varying the nature of the dried liver fractions used did not stimulate growth. When these diets were enclosed in gelatin capsules and force fed to the fish, a marked gain in weight was obtained. Supplementing the meatless diets with gelatin resulted in satisfactory initial growth. The supplemented diets did not sustain continuing growth beyond 7 weeks suggesting that further deficiencies exist in these rations.

14530

Studies on Certain Filtrable Viruses. VI. Antigenic Properties of Entire Embryo Fowl Pox Vaccine.

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In a previous report,¹ the results of immunizing farm flocks with fowl pox vaccine prepared from entire chick embryo were described. The vaccine consisted of a composite of the chorio-allantoic membrane, yolk, yolk sac, albumen, embryo proper, and fluids from previously inoculated embryos that showed gross lesions on the chorio-allantoic membrane. While a high concentration of virus was noted in the chorio-allantoic membrane, the presence of virus in other parts of the embryo was also demonstrated.² The dehydrated vaccine was suspended 1-100 in tryptone broth and applied by the 3-stick method in 35 healthy farm flocks, comprising 25,164 chickens. Nine days after inoculation, more than 6000 birds, approximately one-fourth of the vaccinated birds, were observed with 95.85% showing takes. Forty-three days after inoculation, 71 culled chickens from 3 of the flocks were brought to the Laboratory and artificially exposed to fowl pox virus

by the feather follicle method. Eighty-eight and seven-tenths per cent proved refractory.

The present note concerns an observation on the duration of the immunity produced by entire embryo vaccine. Seventy-one birds selected from 4 of the flocks were exposed, one year subsequent to vaccination, on the patau-gium by the 3-stick method to fowl pox virus in a 1-100 suspension in tryptone broth. The

TABLE I.
Artificial Exposure of Chickens Vaccinated One Year Previously with Entire Embryo Fowl Pox Vaccine.

Flock	Birds exposed* 8/18/43	Reaction to exposure		
		8/25/43	9/1/43	9/9/43
1	35	—	—	—
2	12	—	—	—
3	15	+	+	+
		(3)†	(3)†	(1)†
4	9	—	—	—
Total	71	+	+	+
		(3)	(3)	(1)
Nonvaccinated controls	10	+	+	+
		(10)	(10)	(5)

* Fowl pox virus 3-stick method.

— = No lesion.

+

† No. of birds.

¹ Thorning, W. M., Graham, Robert, and Levine, N. D., *Am. J. Vet. Res.*, 1943, **4**, 250.

² Thorning, W. M., Thesis in partial fulfillment of Master of Science degree, University of Illinois Graduate School, 1942.

exposing virus was of the same strain as that used for vaccination, but consisted of the chorio-allantoic membrane which represents a greater concentration of virus than the entire embryo vaccine. The artificially exposed chickens were given three inspections at weekly intervals for lesions of fowl pox following exposure. Results of the exposure are noted in Table I.

Out of the 71, 3 susceptible birds were

encountered. These were from the same flock. Ten unvaccinated control birds simultaneously exposed to the challenging virus proved susceptible. Though the number of birds included in this observation is too small to permit conclusion, it appears that a large proportion, 95.7%, of this group immunized with entire embryo vaccine proved immune one year after vaccination.

14531

Relation of Changes in Proximal Segment of Renal Nephron to Maintenance of Acid Base Equilibrium.*

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In a previous publication¹ in which consideration was taken of the stability of the acid-base equilibrium of the blood in animals falling in different age periods, the observation was made that in a group of senile animals varying in age from 9-19 years, 6 animals showed chemical evidence of renal dysfunction in which this equilibrium was stabilized against the influence of a period of anesthesia from chloroform lasting for 45 minutes. Other members of this group (Group III) of senile animals which gave no chemical evidence of a renal injury failed to show this factor of resistance to the action of the anesthetic body. Further studies in senile animals have strengthened this observation by an investigation of 23 additional dogs that gave evidence of their ability to maintain what they had established as their normal acid-base equilibrium when this fundamental balance was subjected to an influence which in other senile animals and in dogs of younger age periods resulted in a depletion of the alkali reserve of the blood.

The control group of animals for this study is represented by 29 senile dogs that have

had normal urines both chemically and microscopically, an average elimination of phenolsulphanephthalein of 38% in an hour period and a normal alkali reserve of the blood which varied between 8.05-8.15. Kidney tissue was removed from 4 of these animals under local anesthesia supplemented by nitrous oxide-oxygen. Such tissue has shown histologically unevenly distributed evidence of a chronic glomerular injury without any cytological evidence of epithelial participation in this process. The epithelium of the nephron in its different segments was of normal configuration. The brush border of the cuboidal cells in the proximal convoluted segment remained intact. In these 4 dogs and in the other members of the senile group of controls that gave no chemical evidence of renal disease the use of chloroform for 45 minutes resulted in an edema of the epithelium in the proximal convoluted segment, more rarely vacuolation and fatty degeneration and a disappearance of the brush border of these cells. Associated with such cytological changes in this epithelium there have occurred two modifications in renal function: a rapid reduction in the reserve alkali of the blood to readings as low as 7.75, a delay or inability on the part of the animals to restore this equilibrium and a reduction to as low as 5%

* This investigation was made possible by a grant from the Josiah Macy, Jr., Foundation.

¹ MacNider, Wm. deB., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 1.

in the elimination of phenolsulphanephthalein.

In contrast with these control animals, 23 senile dogs showing chemical evidence of renal dysfunction have been studied by employing a similar experimental procedure. From 4 of these dogs biopsy material was obtained from the kidney by the method which has been outlined. This tissue has also shown a diffuse but unevenly distributed glomerular injury. The order of change has been similar to that for the control senile animals that gave no chemical evidence of renal disease. It is impossible to evaluate comparatively either the severity or the functional significance of the glomerular injury in the two groups of animals. The essential cytological difference in the kidneys as a whole is that in those senile animals which manifested evidence of renal disease there has developed a change in the morphology of the epithelium lining the proximal convoluted segment of the nephron from the normal cuboidal type of cell with a brush border to an epithelium without this specialization and which is imperfectly differentiated into cell units. Such epithelial tissue is of a low, flattened order with prominent deeply staining nuclei and a cytoplasm which stains evenly and intensely. Functionally such animals have established as their pathological normal an alkali reserve of the blood which is below the normal average for such a value: 8.0-7.9. The elimination of phenolsulphanephthalein in an hour period has shown a reduction varying from 18-29%. When such senile animals are subjected to an anesthesia from chloroform for 45 minutes the acid-base equilibrium with 2 exceptions has remained uninfluenced by the anesthetic body. In the 2 exceptions noted the reserve alkali has been reduced from the low normal of 8.0 to 7.9. The elimination of phenolsulphanephthalein in an hour period following the anesthesia has shown a greater degree of variation. In 19 of the dogs the percentage elimination of this dye was unchanged. In 4 of the animals the output was reduced from a maximum of 38% to as low as 12%. Two of these animals with an elimination of phenolsulphanephthalein of 12% and of 17% have been referred to as animals in which the reserve alkali of the blood was also depleted by the anesthetic.

The histological study of the kidneys of these 23 senile animals that showed resistance to chloroform by maintaining the acid-base equilibrium of the blood and which had continued to eliminate phenolsulphanephthalein in amounts equivalent to the preanesthetic percentage demonstrated the inability of the anesthetic to induce injury to the atypical order of epithelium which had made its appearance in the proximal convoluted segment of the nephron. By employing such histological criteria it is impossible to state whether or not the use of the anesthetic altered functionally the effectiveness of the glomeruli. There has been no cytological evidence of such a superimposed acute injury.

In past years this same order of fixed tissue cell resistance associated with morphological change in cell type has been observed for the kidney² and liver³ against secondary injury from uranium nitrate and also for the liver⁴ to the hepatotoxic action of chloroform after a repair process had developed which was associated with a shift in cell type. Furthermore, in certain senile dogs⁵ there has been observed to occur, associated with the aging process, a change in form of the epithelium of this organ from the usual polyhedral type of cell to an order of cell or syncytial structure definitely comparable to the altered type of cell which has been described in this discussion as occurring in the proximal convolution of the nephron in the animals which manifested the same type of resistance to chloroform.

From the comparative study of the 2 groups of senile dogs it would appear that the group which has shown evidence of functional renal impairment and in addition to the chronic glomerular changes common to both groups has shown an alteration in the type of epithelium in the proximal convolution of the nephron, that there is a relationship between this latter change and the ability of such

² MacNider, Wm. deB., *J. Exp. Med.*, 1929, **49**, 411.

³ MacNider, Wm. DeB., *J. Pharm. and Exp. Therap.*, 1936, **56**, 359.

⁴ MacNider, Wm. deB., *J. Pharm. and Exp. Therap.*, 1936, **56**, 373.

⁵ MacNider, Wm. deB., *J. Pharm. and Exp. Therap.*, 1936, **56**, 383.

animals to maintain a normal acid-base equilibrium of the blood and to eliminate phenol-sulphanephthalein.

Structural modification associated with the ageing process which may or may not be designated disease, has resulted in the stabilization of a fundamental equilibrium of the animal organism as the animal relates itself to its normal environment and to an abnormal environment brought about by the introduction of a chemical body foreign to its tissues.

Summary. Twenty-three senile dogs showing varying degrees of glomerular injury and at the same time a fairly diffuse change in the type of epithelium in the proximal segment of the renal nephron from a normal order of cuboidal cell to one of a flattened, less spe-

cialized type of cell when subjected to the anesthetic action of chloroform for 45 minutes fail to show, as the result of the use of this body, a reduction in the elimination of phenol-sulphanephthalein, or a reduction in the reserve alkali of the blood from that value which these animals have established as their pathological normal. In a group of 29 control senile animals, with a normal urine, both chemically and microscopically, with the same order of change in the glomeruli but with, in general, a normal order of cytological structure in the proximal segment of the renal nephron when anesthetized for 45 minutes with chloroform show a marked decrease in the elimination of phenolsulphanephthalein and a reduction in the reserve alkali of the blood.

14532

Effect of Rutin on Increased Capillary Fragility in Man.

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Rutin is, like hesperidin, a flavone glucoside, and the two are very similar in structural arrangement. The chemical formula of rutin is $C_{27}H_{30}O_{16} \cdot 3H_2O$. Hesperidin has been shown by Szent-Györgyi and others¹ to have a beneficial effect on abnormally fragile capillaries, at least in certain cases. It seemed possible that rutin might have a similar effect. So far as we know, no studies have been reported previously in which rutin has been given to man.

Rutin is a constituent of a variety of plants,

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¹ Armentáno, L., Bensáth, A., Béres, T., Ruzsnyák, I., and Szent-Györgyi, A., *Deutsche med. Wchnschr.*, 1936, **62**, 1325.

² Sando, C. E., and Lloyd, J. U., *J. Biol. Chem.*, 1924, **58**, 737.

including tobacco, garden rue, forsythia, elder flowers, and violets. That used in this study was extracted from tobacco by essentially the same process described by Sando and Lloyd.² It had the proper melting point, and its purity and identity were confirmed by carbon and hydrogen determinations and hydrolysis by sulfuric acid to quercetin, dextrose, and rhamnose. The tobacco used was of the flue-cured type, of high quality, cured but not aged or fermented. Lower grades of flue-cured tobacco, the stems of flue-cured tobacco, and air-cured tobacco of any grade, were found to yield little or no rutin.

Rutin was given to 14 patients with hypertension who were attending the cardiac clinic of this hospital. The patients were chosen because they showed an increased capillary fragility, as measured by the Petechial Index of Gothlin.³ The substance was given by

³ Ahlborg, N. G., and Brante, G., *Act. Med. Scand.*, 1940, **104**, 527.

mouth in capsules, starting with 20 mg twice a day and, usually, increasing to 3 times a day in a few weeks. Measurement of the Gothlin Index was repeated every 3 or 4 weeks, not oftener, because Gothlin had pointed out that a negative test is only significant if an interval of at least 3 weeks had elapsed since the previous test. Two of the 14 subjects were receiving potassium thiocyanate by mouth at the time the study was begun, and this was continued.

Results. In no case did any toxic effect develop that could be attributed to the rutin. One patient died of a stroke 2 weeks after beginning the medication. Two other patients did not return for adequate follow-up. However, 11 patients were followed for a period varying between 12 and 16 months. Capillary fragility became normal in 8 of these

within 2 months of beginning medication, while in 3 it remained increased. One of the subjects in whom the fragility remained high developed a hemiplegia 4 months after beginning medication, and is reported to have died. No complication developed during the period of observation in the remaining 10 subjects, nor was there any significant change in blood pressure. Two subjects stopped taking the rutin after the capillary fragility had returned to normal. Within 6 weeks the capillary fragility was again increased. Rutin was resumed, and when the capillary fragility was tested a month later, it was again normal.

Conclusion. At least in certain cases, rutin appears to have the property of decreasing capillary fragility in subjects in whom this fragility is initially increased. In this effect it resembles hesperidin.

14533

Further Observations upon the Protracted Effect of Alpha-tocopherol Administered to Nursing Rats.*

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In a previous paper,¹ it was shown that a single dose of 0.5-5.0 mg of alpha-tocopherol administered on the 15th day of life to rats born of mothers on an E-deficient diet, and maintained after weaning upon vitamin E low diet, produced a significant delay in post-pubertal degeneration of the testis. Similar doses given on the 5th day, or on the 28-30th day, were less effective than when given on the 15th day.

This paper is concerned with the effect of larger single doses, either given to the young directly, or through the mother's milk.

Experiment I. The mother was a third generation rat on E-deficient diet, 10 mg of alpha-tocopherol acetate having been given

at the beginning of each pregnancy. Litter H₂ was the second litter; the first litter of 7 young did not survive 4 days. The young of H₂ were given 1 drop (25.6 mg each) of alpha-tocopherol acetate on the 15th day of lactation. Semi-castration was performed when they were 107 days old, and they were killed on the 182nd day. Repeated fertility tests were made by mating the rats with females which had been kept on a stock diet. During the mating period, male and female had access only to vitamin E-deficient diet. The birth of a litter was considered a positive test.

Experiment II. Each suckling rat of a second litter was given 51.2 mg of alpha-tocopherol acetate on the 15th day. The mother was again a third generation rat on E-deficient diet. The results are summarized in Table II.

From previous data, it has been found that

* Aided by a grant from the John and Mary R. Markle Foundation.

¹ Kaunitz, H., Pappenheimer, A. M., and Schogoleff, C., *Am. J. Path.*, 1944, **20**, 247.

TABLE I.
Effect, upon Testes, of 25.6 mg Alpha-tocopherol Acetate Administered on 15th Day.

Rat No.	Age (days)	Body weight (g)	Testicular weight (g)	Motile sperm	Age at last positive mating (days)	Histology of testis (Mason) ²
H ₂ (1)	107	370	1.478	+		0-I
	182	388	0.744	0	127	IV-V
H ₂ (2)	107	405	1.382	+		0
	182	414	0.766	0	111	IV-V
H ₂ (3)	107	388	1.385	+		0
	182	400	0.800	0	123	III-V
H ₂ (4)	107	390	1.300	+		0
	182	394	0.870	0	150	III-IV

TABLE II.
Effect, upon Testes, of 51.2 mg Alpha-tocopherol Administered on 15th Day.

Rat No.	Age (days)	Body weight (g)	Testicular weight (g)	Motile sperm	Age at last positive mating (days)	Histology of testis (Mason) ²
H51 (2)	202	430	1.538	+		0
	229	415	1.478	0	149	0-I
H51 (3)	202	418	1.460	+		0
	229	400	1.064	0	187	I-III
H51 (4)	202	340	1.400	—	134	0-I
H51 (5)	202	320	1.244	+		0
	229	344	1.134	—	187	0-I

the average weight of one testis in untreated animals under otherwise identical experimental conditions is as follows:

75 days—36 animals—0.930 g
95 " —26 " —0.657 "
110 " —5 " —0.520 "
135 " —11 " —0.490 "

At 75 days, 2 of the 36 untreated control rats had motile sperm, but no positive mating was obtained. After this age, motility was invariably lost and the testis showed advanced degeneration in practically all cases.

It is apparent that the single dose received on the 15th day of life has exerted a prolonged delaying effect upon testicular degeneration. The degree and duration of protection is, with the dose used (0.5 to 51.2 mg), roughly proportional to the amount of tocopherol administered. (Chart 1.)

Experiment III. A single dose of 13 or 26 mg alpha-tocopherol acetate was given to the mothers, either on the 5th or 15th day, so that the only source of vitamin E for the young was the mothers' milk. Again, definite retardation of testicular degeneration resulted, as is shown in Table III.

It is obvious without further analysis that the alpha-tocopherol excreted in the milk has delayed post-pubertal testicular degeneration. Comparing the groups in which the mothers received the single dose on the 5th or on the 15th day, one again notes a more prolonged and favorable effect in the latter group. The larger dose is also obviously more effective than the smaller one.

Reference to the chart suggests that the amount of protection afforded approximates that given by 0.5 to 1.0 mg, in the group where the mother received 13.0 mg on the 15th day, and 2.0 mg in the 26.0 mg group. Since there

² Mason, K. F., *J. Exp. Zool.*, 1926, **45**, 159.

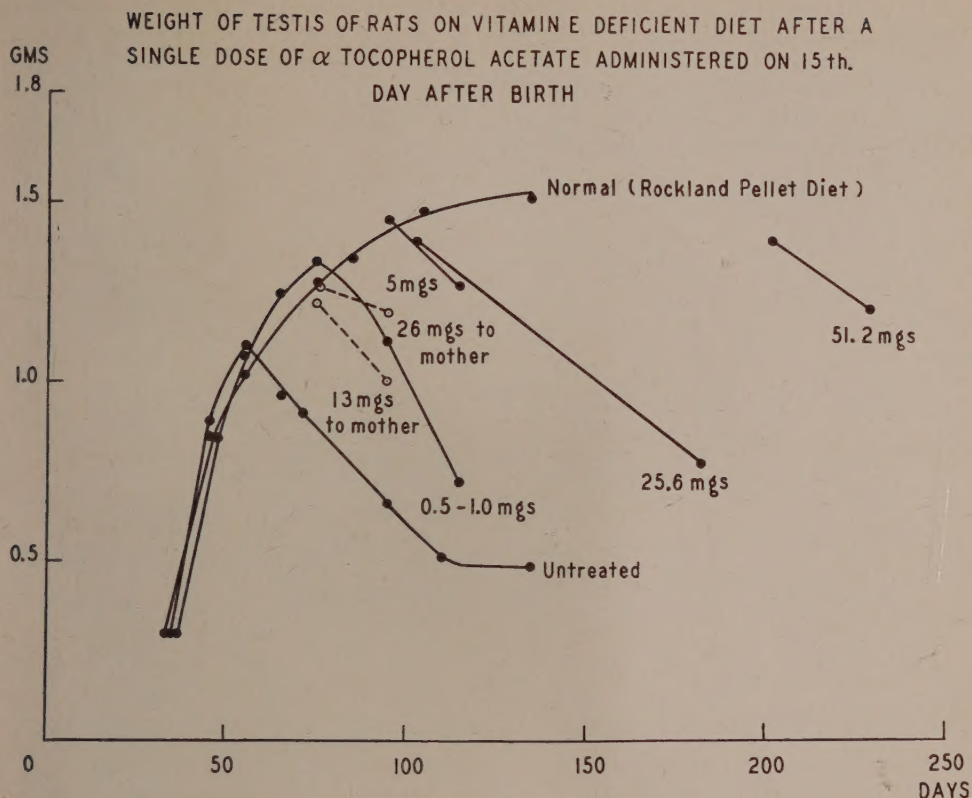


TABLE III.
Effect, upon Testes of Offspring, of Alpha-tocopherol Administered to Mother.

α -tocopherol administered to mother		Testis examined day	No. of experiments	Average weight of testis (g)	Motile sperm		Histology of testis (Mason) ²		
Dose (mg)	Day				No.	%	0-I	II-III	IV-V
13	5	70-75	14	1.235	3	21	6	8	0
13	15	70-75	9	1.242	4	45	5	1	3
26	5	70-75	12	1.357	1	8	10	2	0
26	15	70-75	24	1.285	22	91	24	0	0
Control untreated		70-80	36	0.930	2	5.5	3	20	13
13	5	95	13	0.685	0	0	0	0	13
13	15	95	8	1.004	0	0	3	0	5
26	5	95	11	0.856	0	0	2	1	8
26	15	95	16	1.196	5	31	10	2	4
Control untreated		95	26	0.657	0	0	0	2	24

were 6 individuals in each group, this suggests that at least 50% of the administered tocopherol was excreted in the milk.

Summary. A single dose of alpha-tocopherol acetate, given either to the nursing young or to the lactating mother, delays post-pubertal

degeneration of the testis of rats on vitamin E-deficient diet. This protracted effect is roughly proportional to the amount administered. With a dose of 51 mg, fertility of the offspring is maintained for over 5 months.

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ERRATUM

Article by Meyer, Collins and Marine on Toxicity of Thiouracil, Vol. 55, p. 221, Table I should read g/kg/wt instead of mg/kg/wt.